Transmission dynamics, pathogenicity, and immunological biomarkers of livestock-associated *Staphylococcus aureus*, North Carolina, USA.

by

Pranay R. Randad

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

April 2019

ABSTRACT

Introduction. Animal-adapted *Staphylococcus aureus (S. aureus)*, including methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *S. aureus* (MDRSA), have emerged as an infective agent among pigs raised on industrial hog operations (IHOs) and IHO workers, or those who live near IHOs. IHO workers are at an increased risk of livestock-associated (LA-) *S. aureus* carriage and developing LA-*S. aureus* skin and soft tissue infections (SSTI). The population structure, transmission dynamics, relative pathogenicity, and immunological biomarkers of LA-*S. aureus* contracted by IHO workers and their family members remain poorly understood. **Objectives.** 1) Elucidate the population structure and transmission dynamics of LA-*S. aureus* clonal complex 9 (CC9) between pigs raised on IHOs (IHO pigs) and humans in North Carolina (NC), USA. 2) Determine the degree to which LA-*S. aureus* strains contracted by IHO workers cause disease relative to a highly pathogenic epidemic community-associated (CA-) MRSA strain in a mouse model of SSTI. 3) Develop antigen-specific antibody-based oral fluid (OF) biomarkers and examine their associations with *S. aureus* nasal carriage and SSTI outcomes among a population of IHO workers and their household contacts. **Methods.** 1) 89 LA-*S. aureus* CC9 isolates were subject to bioinformatic and SNP-based phylogenetic analysis and genotyping analyses for antimicrobial resistance (AMR) and virulence factor genes. 2) Measures of pathogenicity – lesion size, innate immune host response, and colony forming units (CFUs) – were compared between mice infected with either LA-*S. aureus* CC9 or CC398 strains to mice infected with CA-MRSA clone SF8300. 3) Develop an OF multiplex *S. aureus* Luminex enzyme immunoassay (EIA) that can be employed in occupational and community settings. Associations between *S.*

aureus nasal carriage and SSTI outcomes and *S. aureus* antigen-specific antibody levels in OF were examined among IHO workers and their family. **Results.** 1) High-resolution phylogenetic and genotyping analysis of LA-*S. aureus* CC9 isolates revealed two distinct transmission clusters which contained IHO pig and human isolates with a high degree of phylogenetic relatedness. Transmission cluster isolates were enriched with multiple acquired AMR genes and a MDRSA phenotype. 2) Mice infected with CC398 LA-*S. aureus* strains developed larger lesion sizes with higher bacterial burden than mice infected with CA-MRSA (USA300 clone, SF8300). The LA-*S. aureus* infected mice had decreased IL-1β protein levels compared to CA-MRSA-infected mice, suggesting a suboptimal host response to LA-*S. aureus* SSTIs. 3) An OF multiplex *S. aureus* EIA was developed. OF IgG antibody levels against SCIN, ClfA, and AT were elevated among adults compared to children. OF IgG levels waned with aging, whereas IgA levels remained elevated independent of age. Anti-ClfA IgA and IgG antibody levels were elevated among IHO workers who carried *S. aureus*, multidrug-resistant *S. aureus* (MDRSA), and tetracycline-resistant *S. aureus* (tet[R]-*S. aureus*) intranasally. Anti-ClfA IgA levels were positively and anti-SCIN levels negatively associated with self-reported SSTI. **Conclusions.** 1) LA-*S. aureus* CC9 in the USA is distinct from lineages in Europe and Asia, and may be transmissible between IHO pigs and humans. Pig-human transmission isolates were associated with the MDRSA phenotype and a greater number of acquired AMR genes. 2) LA-*S. aureus* CC398 and CC9 display an equivalent or greater degree of pathogenicity compared to CA-MRSA USA300, SF8300, in a mouse model of SSTI. 3) OF antibodies against ClfA may serve as non-invasive biomarkers of *S. aureus* colonization and SSTI among IHO workers and their household contacts.

Research Committee Members:

Dr. Christopher D. Heaney,[#]* Associate Professor, Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health

Dr. Alan Scott,*[$] Professor, Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health

Dr. Lloyd Miller,* Associate Professor, Dermatology, Johns Hopkins School of Medicine

Dr. Meghan Davis,* Assistant Professor, Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health

Dr. Fenna Sille,^ Assistant Professor, Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health

Dr. Aaron Milstone,^ Associate Professor, Infection Disease, Johns Hopkins School of Medicine

Dr. Robert Gilman,^ Professor, International Health, Johns Hopkins Bloomberg School of Public Health

[#] = PhD advisor

[$] = Final oral exam committee chair

* = Served on final oral exam

^ = Alternate committee members

Contents

LIST OF TABLES

Table 3M.2. Preliminary association between *S. aureus* nasal carriage status and oral fluid IgA and IgG antibody levels against SCIN, ClfA, ClfB, AT, and BT. (142)

LIST OF FIGURES

INTRODUCTION

Staphylococcus aureus is a leading cause of human bacterial infections in the United States (USAUSA), and can cause both superficial and invasive infections, including sepsis, bacteremia, abscesses, pneumonia, osteomyelitis, endocarditis, and meningitis.[1] This pathogen is also a common human commensal organism carried asymptomatically in the nares of ~30% of the human population,[2] which is noteworthy because nasal carriage of *S. aureus* is associated with *S. aureus* infection[3]. The emergence of methicillin-resistant *S. aureus* (MRSA) has made treatment of these infections difficult, and increasing resistance to multiple antibiotics, giving rise to multidrug-resistant *S. aureus* (MDRSA), has further complicated this situation.[3,4] In the United States, invasive infection with MRSA is associated with an in-hospital case fatality rate of 13%.[5] While the Center for Disease Control and Prevention (CDC) reports a decrease in hospital-associated (HA) MRSA infections in the USA since 2009, largely due to the successful uptake of hospital infection control measures,[5] community-associated MRSA (CA-MRSA) and methicillin-susceptible *S. aureus* (CA-MSSA) infections have only marginally decreased or remained steady since 2009.[5,6] In contrast to HA-*S. aureus* infections that occur among individuals with predisposed hospitalization risk factors or co-morbidities, CA-*S. aureus* occurs independent of exposure to the hospital environment among otherwise healthy individuals.[7] CA-*S. aureus* is now a health problem in nearly all industrialized countries, and is prevalent and widespread as both an asymptomatic human colonizer and symptomatic pathogen.[8] In particular, skin and soft tissue infections (SSTI) represent a majority of CA-MRSA infection cases,[3] and

it has been estimated that up to 50% of SSTI's in the USA are attributable to CA-MRSA.[8] Thus, the reservoirs and drivers of antimicrobial resistant *S. aureus* outside of the hospital setting, and the dissemination of these pathogens into human communities, represent a major and urgent public health concern.

In the past decade it has become evident that animal-adapted *S. aureus*, including MRSA and MDRSA, have emerged among pigs raised on industrial hog operations (IHOs) and among humans who have frequent contact with, or live in close proximity to IHOs both globally and in the USA.[9–11] Price et al. provided a framework for the bidirectional transmission of livestock-associated (LA-) *S. aureus* between pigs and humans: antibiotic-susceptible LA-*S. aureus* of human origin may be contracted by IHO pigs, where the use of antibiotics created a selective pressure for methicillin-resistant and tetracycline-resistant LA-*S. aureus* that could then disseminate back into human populations.[12] In certain regions of the world there is evidence that LA-*S. aureus* has successfully disseminated into the human community and become a major contributor to human morbidity—in areas of Europe, LA-*S. aureus* accounts for up to 25% of MRSA cases.[13] Consistent with these observations, studies demonstrate that IHO workers in the USA are at an increased risk of carrying LA-*S. aureus,* including LA-MRSA and LA-MDRSA,[10,14] and developing LA-*S. aureus* skin and soft tissue infections (SSTI).[15,16] However, the state of knowledge in the USA is less developed than it is in Europe. In North Carolina (NC), evidence suggests that LA-*S. aureus* originating at IHO's can be transmitted between IHO workers and their household contacts, and that the prevalence of MDRSA and MRSA carriage among children living in the same household as IHO workers was greater than that of children living in households with adults with no known

livestock exposure in the same communities.[10] More recent studies have begun to uncover environmental routes of exposure,[17] raising questions about the extent to which community residents might be exposed to airborne LA-*S. aureus* derived from IHOs.[18]

The transmission dynamics and extent to which LA-*S. aureus* strains contribute to human disease represent critical knowledge gaps in the USA, which until filled, will hinder efforts to control and prevent the dissemination of LA-*S. aureus* into human populations. Furthermore, the development and application of *S. aureus*-specific, non-invasive, immunological biomarkers to measure early biologic effects of *S. aureus* exposure, colonization, and infection among IHO worker populations remains entirely unexplored. The dissemination and pathogenic potential of LA-*S. aureus* in humans has been met with skepticism, with reports suggesting that these strains are transient nasal colonizers of IHO workers, display a decreased capacity for human-to-human transmission, and are less pathogenic than typical CA- and HA-*S. aureus* strains.[12,19–22] Nevertheless, LA-*S. aureus* strains of pig origin have indeed been reported to produce active and symptomatic SSTIs in humans in USA,[16,23] Canada,[24] and Europe.[12,13,25,26] While epidemiological studies have concluded that IHO workers face occupational exposure to *S. aureus,*[14,17] including MRSA and MDRSA, the development and application of immunological biomarkers of *S. aureus* colonization and infection has largely focused on high-risk populations in the clinical setting.[27] As our efforts to understand and combat the community origins of antimicrobial-resistant *S. aureus* infections become a priority, it is critical that we examine the transmission dynamics and pathogenic potential of LA-*S. aureus* that have contributed to human morbidity in parts of the world and are now raising concern in parts of the USA Furthermore, there is a need

to develop non-invasive immunological biomarkers that can be employed in community and occupational settings, to target and intervene on human exposure, colonization, and infection with these emerging multidrug-resistant LA-*S. aureus* strains.

This dissertations aims to fill the following three (3) critical knowledge gaps:

1. While LA-*S. aureus* clonal complex (CC) 398 (CC398) appears to be the major circulating LA-*S. aureus* clone in Europe,[12,25] LA-*S. aureus* CC9 has emerged as an important LA-*S. aureus* clone in Asia.[28–30] Epidemiologic and whole genome sequence analysis (WGSA-) based studies have provided evidence of the population structure and transmission dynamics of LA-*S. aureus* CC398 between pigs and humans,[25] and an increasing prevalence of LA-*S. aureus* CC398 SSTI's and blood stream infections (BSIs) in parts of Europe.[31] LA-*S. aureus* CC398 has also been reported to cause SSTI among IHO workers in the USAUSA.[16,32] Especially concerning is that LA-*S. aureus* CC398 is largely methicillin or multidrug-resistant, which has been attributed to the use of antibiotics in IHOs.[12] While multiple studies have identified IHO worker populations with a high prevalence of LA-*S. aureus* CC9 nasal carriage,[14] and some cases of SSTI-associated LA-*S. aureus* CC9[15] in the USA, no studies, to our knowledge, have employed WGSA to investigate the population structure and transmission dynamics of LA-*S. aureus* CC9 between IHO pigs and humans in the USAUSA **Chapter 1 of this dissertation aims to elucidate the population structure of LA-*S. aureus* CC9, and provide evidence of transmission of LA-*S. aureus* CC9 between IHO pigs and human in NC, USA, a region with a particularly high density of IHOs**.[33]

2. The majority of CA-*S. aureus* infections in the USA involve skin and soft tissue.[3,34–36] USA300 is one of the most common CA-*S. aureus* clones recovered from individuals presenting to hospitals with SSTIs.[36] USA300 is a CA-*S. aureus* clone of reputable pathogenicity with the capacity to cause unusually severe SSTI disease.[37] These observations have been supported by and are consistent in mouse models, with reports concluding that CA-MRSA strains, including USA300 clone SF8300, are significantly more virulent than HA-MRSA in both a mouse model of bacteremia and SSTI.[38] Apart from a single study suggesting that LA-*S. aureus* displays greater lethality than human-associated *S. aureus* in a murine sepsis model,[39] there is a paucity of data regarding the phenotypic pathogenicity of predominant LA-*S. aureus* lineages contracted by IHO workers and implicated in human SSTI. No studies, to our knowledge, have attempted to compare the pathogenic potential of LA-*S. aureus* CC398 and CC9 of IHO origin to a CA-MRSA infection strain of reputable pathogenicity – i.e. USA300 clone SF8300[37] – in a mouse model of SSTI. **Chapter 2 of this dissertation aims to understand the degree to which LA-*S. aureus* strains contracted by IHO workers cause disease relative to an epidemic CA-MRSA strain of high pathogenicity (USA300 clone SF8300) in a mouse model of SSTI.**

3. The *S. aureus* genome encodes for approximately 2700 proteins.[40] The human adaptive immune response can serve as a sensor of *S. aureus* protein expression in vivo, which is recorded and maintained in the profile of human antigen-specific antibody levels directed against *S. aureus*-specific proteins. Previous studies have characterized important associations between serum IgA and IgG antibody levels

against various *S. aureus* proteins and outcomes of *S. aureus* colonization and infection in diverse healthy populations,[27] hospitalized adults,[27,41] adults with invasive *S. aureus* infections,[27,42] dialysis patients,[27] intravenous drug-users,[42] and children colonized or presenting with *S. aureus* infections.[43] All of these studies measured IgA and IgG antibody levels in serum. To our knowledge, no studies have developed non-invasive antibody-based biomarkers of *S. aureus* colonization and infection in oral fluid (OF) that could be employed in occupational and community populations, particularly in children, from whom blood collection may be challenging. Furthermore, no studies, to our knowledge, have aimed to measure *S. aureus* antigen-specific IgA and IgG antibody levels in OF among a population of IHO workers who experience unique occupational exposure to LA-*S. aureus*.[14] **Chapter 3 of this dissertation aims to develop antigen-specific antibody-based OF biomarkers and examine their association with *S. aureus* nasal carriage outcomes and SSTI among a population of IHO workers and their adult and child (7-17 years of age) household contacts.**

It is anticipated that this dissertation will provide significant contributions to understanding of the transmission dynamics of LA-*S. aureus* between IHO pigs and humans and pathogenic potential of LA-*S. aureus* commonly contracted by IHO workers in NC, USA. Furthermore, this dissertation will advance the development and application of non-invasive immunological biomarkers of *S. aureus* colonization and infection that could be employed broadly in occupational and community population settings where frequent, repeated blood-based sampling presents challenges for surveillance.

CHAPTER 1

Livestock-associated (LA-) *Staphylococcus aureus* clonal complex (CC) 9: Population structure and evidence of transmission between pigs raised on industrial hog operations and humans in the United States

ABSTRACT

Introduction. Livestock-associated (LA-) *S. aureus* clonal complex (CC) 398 predominates in Europe, whereas LA-*S. aureus* CC9 has emerged as an important lineage in Asia and the USA. While whole genome sequence analysis (WGSA) provided robust evidence of the population structure and transmission dynamics of LA-methicillin (LA-MRSA) and LA-multidrug-resistant (LA-MDRSA) *S. aureus* CC398 between pigs and humans in Europe, other LA-*S. aureus* strains, particularly CC9, remain poorly characterized.

Objectives. To evaluate the population structure and transmission dynamics of LA-*S. aureus* CC9 in industrial hog operation (IHO) pigs and individuals who work in or live near IHOs.

Methods. Forty nine LA-*S. aureus* CC9 collected from IHO pigs, IHO workers, adult and child (7-17 years) household contacts of IHO workers, and community referent (CR) adults and their child household contacts (households with no known exposure to livestock) living in the top 10 hog-producing counties in North Carolina (NC), USA, between 2013-2017, were prepared for sequencing. Along with whole genome sequence data from 32 LA-*S. aureus* CC9 isolates representing an international collection, a total of 81 LA-*S. aureus* CC9 were subjected to bioinformatic analyses. Transmission clusters, defined as IHO pig and human LA-*S. aureus* CC9 isolates separated by 42 or fewer SNPs, were identified.

Results. Phylogenetic analysis demonstrated three major lineages of geographically restricted LA-*S. aureus* CC9, of which a single lineage contained 100% of the NC LA-*S.*

aureus CC9 isolates (Lineage III). High-resolution phylogenetic and genotyping analysis of Lineage III isolates revealed multiple distinct sub-lineages of LA-*S. aureus* CC9, two of which contained transmission clusters with a high degree of phylogenetic relatedness between IHO pig and human isolates. Transmission cluster isolates carried a statistically significantly higher number of acquired antimicrobial resistance (AMR) genes and a higher proportion were MDRSA, compared to non-transmission cluster isolates.

Conclusions. This study suggested that LA-*S. aureus* CC9 isolates from humans and IHO pigs in NC originate from a common pool, and provided evidence of transmission of LA-*S. aureus* CC9*,* including LA-MDRSA, between IHO pigs, IHO workers, and community referent adults with no known exposure to livestock in a region in the USA with intensive hog production.

INTRODUCTION

The intensive use of antimicrobials in food animal production may create a selective pressure that gives rise to antimicrobial resistant (AMR) pathogens. Thus, industrial hog operations (IHO) may be reservoirs of occupational and community exposure to antimicrobial resistant (AMR) pathogens.[44] In particular, livestock-associated (LA-) *Staphylococcus aureus* (LA-*S. aureus*) has emerged among swine raised in IHOs (hereafter IHO pigs) and individuals who live near or have frequent contact with pigs raised in IHOs globally, including the United States (USA) .[9–11] Consistent with these observations, IHO workers who are occupationally exposed to pigs are at increased risk of carrying *S. aureus* intranasally, including methicillin-resistant *S. aureus* (MRSA), multidrug-resistant *S. aureus* (MDRSA), and LA-*S. aureus.*[14,16] Furthermore, individuals exposed to LA-*S. aureus* are at risk of developing mild-to-severe infections, including skin and soft tissue infections (SSTIs), pneumonia, endocarditis, osteomyelitis, and bactermia.[15,16,25,32] *S. aureus* CC398 appears to be the predominant LA-*S. aureus* lineage circulating in Europe[12,25] and some areas of the USA – e.g., Iowa.[45] Since it was first reported in the early 2000's,[46] zoonotic LA-*S. aureus* CC398 is an increasing cause of human infection in the general population of several EU nations.[25,32] More recently, it has become clear that diverse clones may be emerging in association with IHOs. For example, *S. aureus* CC9 has been reported as a dominant LA-*S. aureus* lineage in Asia and it has been described as an emerging clone in some areas of the US with intensive industrial livestock production.[28,29]

While whole genome sequence analysis (WGSA) studies have provided robust support for the transmission of LA-MRSA and LA-MDRSA CC398 between swine and

humans in Europe,[12,14,16,25,32] the population structure and transmission dynamics of emerging LA-*S. aureus* strains, particularly CC9, remain poorly understood in the USA. To our knowledge, no studies in the USA have employed WGSA to elucidate the global and regional transmission dynamics of LA-*S. aureus* CC9. Previous epidemiologic studies in North Carolina – the 2nd leading pork producing state in the USA (behind Iowa) – showed a high prevalence of LA-*S. aureus* CC9 among pigs raised on IHOs (IHO-pigs)[17] and IHO-workers.[14] Epidemiologic findings also provided support for transmission of LA-*S. aureus* CC9 between IHO-workers and their household contacts,[14] and identified some instances of LA-*S. aureus* CC9 occurrence among community residents with no known exposure to livestock in high density IHO areas of NC (hereafter community referent [CR] adults) .[10] However, no studies of this emerging LA-*S. aureus* CC9 clone have employed WGSA to characterize the population structure and transmission dynamics in a region of intensive industrial hog production in the USA. The objectives of this study were to employ whole genome sequencing and phylogenetic analysis to: 1) elucidate the population structure of LA-*S. aureus* CC9 collected from various regions in North America, South America, Europe, and Asia; 2) characterize the transmission dynamics of LA-*S. aureus* CC9 collected from IHO pigs, IHO workers, children of IHO workers (IHO child), and CR adults in NC, USA.; and 3) examine whether the antimicrobial use (AMU) plays a role in driving the clonal expansion of LA-*S. aureus* CC9 in the USA, represented by the frequency of AMR genes within transmission clusters.

METHODS

S. aureus *isolate selection from humans.*

S. aureus isolates were collected from participants who were previously enrolled into one of three separate epidemiologic studies and screened for nasal carriage of *S. aureus*. The first study (Study 1) was cross-sectional and surveyed 204 IHO workers for *S. aureus* nasal carriage at a single time point.[9] The second study (Study 2) was longitudinal and followed 183 IHO workers and their adult and child (7-17 year) household contacts for 4 months, and assessed *S. aureus* nasal carriage at baseline and 8 biweekly (once every two weeks) time points.[14] The third study (Study 3) was cross-sectional and assessed *S. aureus* nasal carriage among 800 participants living in the top 10 hog producing counties of NC; approximately half were household comprised of one IHO worker and one child (<7 years) [IHO child]) and the remainder were households comprised of one CR adult and one CR child (<7 years) who had no known exposure to livestock.[10] Questionnaires were used to collect demographic information, household-level characteristics, and habitual activities (including occupational activities) that could be related to *S. aureus* exposure. For human LA-*S. aureus* CC9 isolates, one of each unique CC9-associated *spa* type (t337, t1415, t3446, t3270, t1419), per individual was selected for sequencing. For Study 2, the longitudinal study, an IHO worker must have carried a putative *S. aureus* CC9 isolate at two time points, or have had a household contact who carried a putative *S. aureus* isolate at one time point, to be included in the study. For Study 1, Study 2, and Study 3, a BD BBL™ CultureSwab™ was used to collected nasal swab samples from both nares of each participant. For Study 1, Swabs were inoculated into 10ml of Mueller-Hinton broth containing 6.5% NaCl (MHB+NaCl)

and incubated overnight at 37ºC. A loop of the enriched media was then streaked onto CHROMagar™ Staph aureus (CA) plates (BD, Franklin Lakes, NJ) and incubated at 37ºC for 24 hours. Colonies with morphology characteristic of *S. aureus* were streaked to isolation. For Study 2 and Study 3, 100ul of participants self-collected nasal swab samples were first directly plated on CA and incubated at 37ºC for 24 hours. Swabs resulting in no presumptive *S. aureus* growth following direct plating were processed using methods previously described.[47] Up to two colonies with morphological characteristics of *S. aureus* were streaked to isolation on either CA or tryptic soy agar with 5% sheep erythrocytes (blood agar, Remel).

S. aureus *isolate selection from pigs.*

S. aureus isolates from IHO pigs were collected from a convenience sample of a single IHO in North Carolina (IHO-1), as described previously,[17] or from IHO pig rope samples from 20 IHOs in North Carolina (IHO-2 through IHO-21); the latter have not been previously published. For IHO-1 pig isolates, Copan E-swabs were used for sample collection – each pig was swabbed in the right nares, right side of mouth, skin behind the right ear, right perineal mucosa, and any observed skin lesion as previously described.[17] Pig swabs were subjected to double-enrichment in broth culture using the methods previously described.[17] For IHO-2 through IHO-21, rope sample elution's were processed for *S. aureus* following the same procedure outlined for Study 2 and Study 3. Direct plating of rope sample elutions did not result in any *S. aureus* growth, thus all rope sample isolates included in this study were enriched prior to isolation.

Molecular characterization of* S. aureus *isolates.

The *spa*-type or sequence type (ST) was previously characterized for all *S. aureus* isolates,[9,10,14,17] and used to assign each isolate to a putative multi-locus sequence type (MLST).

Genome sequencing.

A representative sample of LA-*S. aureus* CC9 isolates from the parent studies were sequenced and assembled. For human LA-*S. aureus* CC9 isolates, one of each unique CC9-associated *spa* type (t337, t1415, t3446, t3270, t1419), per individual was selected for sequencing. For IHO-1 pig samples, one of each CC9-associated *spa* type per pig life stage per barn was selected for sequencing. For the IHO pig rope samples, one of each CC9-associated *spa* type per *S. aureus* positive rope was selected for sequencing (each rope corresponded to a single IHO). DNA was prepared for multiplexed, paired-end sequencing on an Illumina MiSeq (Illumina, Inc., San Diego, CA). Libraries for 25 isolates were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, Inc.) according to manufacturer instructions. Libraries for 48 isolates were prepared using the Kapa Hyper Prep Kit (Kapa Biosystems, Inc.), using the recommended adaptor concentrations for libraries constructed from 500 ng input DNA. For libraries prepared using the Kapa Hyper Prep Kit, uniquely barcoded adaptors were obtained from BioO Scientific® (BioO Scientific®, NED, NEXTflex-96™ DNA Barcodes for DNA), and libraries were quantified via quantitative PCR using the Kapa Library Quantification kit (Kapa Biosystems, Inc. Wilmington, MA,). Based on individual library concentrations, equimolar pools of *S. aureus* libraries were prepared at a concentration of 2nM. The pooled libraries were qc'd on an Agilent bioanalyzer and sequenced on an Illumina MiSeq at 2X 300 bp.

Core-genome-based phylogenetic analysis.

To study the genetic relatedness of LA-*S. aureus* CC9 isolates to each other and to an international collection of *S. aureus* CC9, SNPs from the core genome of all NC CC9 isolates along with 32 publicly available *S. aureus* CC9 whole genome sequence datasets (National Center for Biotechnology Information: Reference Sequence Database [RefSeq]; https://www.ncbi.nlm.nih.gov/refseq/), were used to construct a maximum likelihood phylogeny. We closed the genome of the highest quality CC9 sequence from the NC collection, and used this as a reference for alignment. Using the NASP pipeline (v.1.0.0),[48] Illumina short-read sequences were aligned to the reference *S. aureus* CC9 genome by using BWA-MEM (v.0.7.12).[49] SNPs were called using GATK (v.3.5) [50] with the following parameters: ≥10× mapping coverage; ≥90% unambiguously base calls; insertions and deletions were ignored. Recombinant regions were removed using Gubbins (v.2.1) ,[51] and the resulting SNP matrix was used to construct phylogenetic trees in PhyML with Smart Model selection (v.3.0).[52] Support values were calculated by bootstrap sampling (n=100). The methods were then repeated for a subset of clustered *S. aureus* CC9 isolates. Isolates that were collected from NC are referred to hereafter as the "NC collection". Isolates collected from RefSeq are referred to hereafter as the "International collection".

Molecular characterization of virulence factor (VF) genes, and antimicrobial resistance (AMR) genes.

Virulence factor (VF) and Antimicrobial resistance (AMR) genes were determined using the VirulenceFinder 1.5[53] and ResFinder 3.0[54] databases available on the Center for Genomic Epidemiology (CGE) server. Contigs were screened for VF and

AMR genes using ABRicate (https://github.com/tseemann/abricate), a modified BLASTn based tool for the screening of genes in assemblies.

Statistical analysis.

A data driven approach was used to identify epidemiological evidence of clustering, which was then used to define a SNP threshold for identifying putative LA-*S. aureus* CC9 transmission between IHO pigs and humans (hereafter referred to as "transmission clusters"). To examine the relationship between transmission cluster isolates and acquired AMR, we compared the average number of AMR genes per isolate among transmission cluster isolates to non-transmission cluster isolates. Beta coefficients and 95% confidence intervals (CIs) were estimated using a generalized linear model. We compared the prevalence of AMR genes and phenotypic resistance between transmission cluster isolates and non-transmission cluster isolates using a Chi-square exact test.

RESULTS

We screened a total of 1,121 *S. aureus* isolates collected from the nares of 1,187 people enrolled in one of three separate epidemiologic studies in NC, between 2012 and 2016, for LA-*S. aureus* CC9. We assigned a putative CC9 MLST to 145 of these *S. aureus* isolates, based on *spa*-type or sequence type as previously described [14]. Among the 145 putative CC9 isolates collected from humans, 53 representative isolates were selected for whole genome sequencing based on the selection criteria outlined in the methods. A total of 37 *S. aureus* CC9 isolates were collected from 20 pigs from IHO-1, all of which were assigned a putative CC9 MLST.[17] 11 of these isolates were selected for whole genome sequencing (at least one isolate per pig life stage, per barn). Among 20

IHO pig rope samples, representing 20 different IHOs, we recovered a total of 57 *S. aureus* isolates from 5 IHO pig rope samples. 54 of these isolates were assigned a putative CC9 MLST, of which 6 isolates were selected for sequencing (one of each unique CC9-associated *spa* type, per rope). Thus, a total of 70 putative *S. aureus* CC9 isolates were prepared for whole genome sequencing. After removing 21 low-quality genomes, due to either contamination or poor coverage (<2,000,000 bases) at 25x of depth, 49 MLST confirmed LA-*S. aureus* CC9 genomes were sequenced at an average depth of 65.76x (SD = 27.66), using the 2,815,299 base IHOW6 chromosome as a reference. The IHOW6 reference genome had excellent coverage at 25x (2,771,213) and only 1.33% unclassified reads. Among these 49 LA-*S. aureus* CC9 genomes from the NC collection, 39 human isolates were collected from 30 humans, and 10 pig isolates were collected from 5 IHOs. Additional isolate characteristics for these 49 LA-*S. aureus* CC9 isolates are provided in Table 1S.1.

Population structure of LA*-S. aureus *CC9.

A total of 81 LA-*S. aureus* CC9 genome sequences were included in a core genome SNP-based phylogenetic analysis aimed to elucidate the population structure of LA-*S. aureus* CC9 originating from different countries and hosts. This included 49 MLST confirmed, high quality, LA-*S. aureus* CC9 isolates from the NC collection (including the reference), and 32 LA-*S. aureus* CC9 isolates from the International collection. The total LA-*S. aureus* CC9 population exhibited three major lineages (LI, LII, and LIII), that were geographically restricted (Figure 1.1). One of these lineages, LIII, included 100% (49/49) of the LA-*S. aureus* CC9 isolates from the NC collection, which made up 98% (49/50) of LIII isolates (Figure 1.1, Table 1S. 2). LI was composed

of primarily isolates from the Asian continent (92%, 12/13 isolates), 46% (6/13) from China, and 46% (6/13) from Taiwan (Figure 1.1, Table 1S.2). LII was composed primarily of isolates originating from the European continent (100%, 14/14 isolates), 71% (10/14) from Germany, 21% (3/14) from the Netherlands, and 7% (1/14) from Denmark (Figure 1.1, Table 1S.2). LIII was composed primarily of isolates collected from the USA (98%, 49/50 isolates) (Figure 1.1, Table 1S.2). Only two isolates grouped into a lineage that did not correspond to continent of predominance within the ML. A single isolate from Colombia grouped into LIII with isolates from the USA, and a single isolate from the Netherlands grouped into LI with isolates from Asia (Figure 1.1, Table 1S.2). The three (3) ML's of LA-*S. aureus* CC9 appear to correspond to continent of origin, and display little evidence of inter-continental spread. LA-*S. aureus* CC9 from humans and pigs in USA are distinct from the European and Asian lineages.

Presence of Immune Evasion Cluster (IEC) genes.

Absence of the *scn* gene is commonly used as a biomarker of LA-*S. aureus* contracted from pig.[14] A total of five isolates were positive for the *scn* gene (Figure 1S.1). Three were from humans, and two were missing host data. Only a single NC collection isolate carrying the *scn* gene clustered in LI-LIII, and it was contained in LIII (Figure 1.1, Table 1S.3). This isolate was collected from an IHO-worker in NC. The remaining 4 *scn*(+) isolates did not cluster into LI-LIII and were the most distantly related isolates from LI-LIII isolates. These isolates were from the International collection and were collected from the USA, UK, and Taiwan. The *scn* gene is a part of the immune evasion cluster (IEC) carried on the mobile Sa3-prophage.[55] Additional genes that can be encoded on the Sa3-prophage include *sak,* encoding for staphylokinase, and *sea* and *sek*, encoding

for the superantigens staphylococcal enterotoxin A and K respectively.[55] A total of three isolates were positive for the *sak* gene, and were not contained in LI-LIII (Figure 1.1, Table 1S.3). All of the *sak*-carrying isolates also carried *scn.* No isolates were positive for the *sea* or *sek* gene. An absence of IEC genes in LI-LIII isolates suggest that LI-LIII isolates may be members of a larger LA-*S. aureus* CC9 clade.

Presence of acquired AMR genes.

The genetic foundation for the successful expansion of LA-*S. aureus* CC9 in the USA was investigated by comparing the prevalence of AMR genes in LIII (Americas) to LI (Asia) and LII (Europe) respectively. Compared to LIII isolates, LI and LII isolates were enriched for determinants conferring resistance to methicillin and trimethoprim antibiotics (Figure 1.1, Table 1S.3). The *mecA* gene conferring resistance to methicillin was only found in LI and LII. Trimethoprim resistance was encoded exclusively by *dfrG* in LI, and *dfrK* in LII. Determinants conferring resistance to macrolides were evenly distributed in LI-LIII (Table 1S.3). Compared to LIII, LI was enriched with determinants conferring resistance to lincosamides, whereas lincosamide resistance was absent from LII. *lnu(B)* was exclusive to LI, whereas *lnu(A)* was exclusive to LIII (Table 1S.3). Compared to LIII, LI isolates were enriched with determinants conferring resistance to tetracyclines (Table 1S.3). *tet(L)* was the most abundant tetracycline resistance gene in LI, while *tet(K)* and *tet(L)* were present equal proportions in LIII. Compared to LIII, LI isolates were enriched for determinants conferring resistance to aminoglycosides, whereas these genes were absent from LII. The *spc* gene was the most abundant aminoglycoside resistance gene in LIII, and was exclusive to LIII (Table 1S.3). The *ant(6)-la* and *aac(6')-aph(2")* genes were the most abundant aminoglycoside resistance

genes in LI, and *ant(6)-la* was exclusive to LI. The *vga(A)LC* gene encoding resistance to Streptogramin B was exclusive to LIII. Taken together, determinants of resistance to tetracycline, macrolide, lincosamide, and aminoglycoside antibiotics were associated with genetic lineage, and if strains carrying these determinants were enriched through selective pressure, then it is possible that AMU in humans or animals may have played a role in the clonal expansion of LA-*S. aureus* CC9 in the USA.

High-resolution phylogenetic analysis of LIII LA*-S. aureus *CC9.

Because 100% of the NC collection isolates grouped into LIII, we performed a high-resolution core-genome SNP-based phylogenetic analysis on all LIII isolates. A total of 50 isolates were included in the high-resolution phylogenetic analysis. 49 isolates were from IHO-pigs (n=10), IHO-workers (n=34), IHO-minors (n=3), or CR-adults (n=2) in NC, USA. A single (1) isolate was from the international collection, and originated from a pig in Columbia. High-resolution phylogenetic analysis of LIII revealed multiple distinct sublineages, one of which included exclusively IHO-pig isolates (IHO-pig cluster) (Figure 1.2). The maximum SNP-distance among these phylogenetically clustered IHO pig isolates from the same farm (n=6) was used to define putative transmission clusters. The maximum pairwise SNP distance of this IHO-pig cluster was 42 SNP's (range: 2-42 SNP's), and was set as the threshold to classify putative pig-human transmission clusters. Employing this standard, a total of 19 human and pig isolates were separated by 42 SNP's or less, and were included in two distinct transmission clusters (Figure 1.2). The average pairwise SNP-distance between IHO-pig cluster isolates was 24 SNP's (95% CI: 19.33, 28.67), and the average pairwise SNP-distance between transmission cluster isolates was 24.6 SNP's (95% CI: 23.2, 25.9) and

10.8 SNP's (95% CI: 6.67, 14.93) for each distinct transmission cluster. Taken together, a high degree of phylogenetic relatedness between intermingled IHO pig and human transmission cluster isolates strongly suggest that IHO-pig and human LA-*S. aureus* CC9 in NC, USA come from a common pool.

Evidence of transmission of LA-S. aureus CC9 between IHO-pigs and IHO-workers in NC, USA.

Seventy four percent (14/19) of the transmission cluster isolates were collected from IHO workers (Figure 1.2, Table 1S.4). Two IHO-worker isolates differed from an IHO-pig isolate by only twelve SNPs. These results strongly suggest transmission of LA-*S. aureus* CC9 between IHO pigs and IHO workers in NC, USA Notably, an IHO-worker isolate that was associated with a recent skin and soft tissue infection (SSTI) differed from a IHO-pig isolate by only 20 SNPs.

Evidence of transmission of LA-S. aureus CC9 between IHO-workers and IHO-worker household contacts in NC, USA

Sixty six percent (2/3) of IHO child isolates were identical (0 SNP differences) to IHO worker isolates (Figure 1.2), suggesting transmission of LA-*S. aureus* CC9 between IHO-workers and children living in the household of IHO-workers in NC, USA.

Evidence of transmission of LA-S. aureus CC9 to community members with no known exposure to livestock.

One out of the 19 transmission cluster isolates (5%) were collected from a CR adult with no known exposure to livestock (Figure 1.2, Figure 1S.4). The minimum SNP-distance between this isolate and an IHO-pig isolate was 25 SNPs, and between this

isolate and an IHO-worker isolate was 22 SNPs. These results strongly suggest that IHO-derived LA-*S. aureus* CC9 can disseminate into human populations in NC, USA beyond the context of occupational exposure on IHOs.

The role of AMR in transmission of LA*-S. aureus *CC9.

To determine the role of acquired AMR in the transmission of LA-*S. aureus* CC9 between IHO pigs and humans, we compared the average number of AMR genes per isolate between transmission cluster isolates and all other isolates. We detected a significant trend toward an increased number of AMR genes per isolate among transmission cluster isolates compared to all other isolates (beta coefficient = 2.13; 95% CI: 1.31, 2.95) (Table 1.1). On average, transmission cluster isolates carried 3.84 acquired AMR genes per isolate (standard deviation = 1.71) whereas non-transmission cluster isolates carried 1.71 acquired AMR genes per isolate (standard deviation = 1.24) (Table 1.1). Compared to non-transmission cluster isolates, transmission cluster isolates were enriched for determinants conferring resistance to tetracyclines, macrolides, streptogramin B, and lincosamides ($p < 0.05$) (Table 1.2). In particular, transmission cluster isolates were enriched with the *tet(L)* gene conferring resistance to tetracyclines ($p < .05$), a tetracycline resistant phenotype ($p < 0.05$), and a MDRSA phenotype ($p < 0.05$) (Table 1.2).

DISCUSSION

This study is the first, to our knowledge, to provide: 1) evidence for the population structure of LA-*S. aureus* CC9 and 2) a high-resolution phylogenetic analysis that revealed distinct LA-*S. aureus* CC9 sublineages that strongly suggests transmission

of LA-*S. aureus* CC9 between IHO pigs and humans in NC, USA We also found that isolates implicated in transmission between IHO pigs and humans were largely multidrug resistant, enriched with multiple acquired AMR genes. Taken together our results strongly suggests transmission of multidrug-resistant LA-*S. aureus* CC9 between IHO pigs, IHO workers, IHO worker household contacts, and community residents with no known exposure to livestock. Furthermore, our results suggest an important role for AMU in the clonal expansion of LA-*S. aureus* CC9 in NC, USA. An abundance of previous studies have employed WGSA to understand global and regional transmission dynamics of LA-*S. aureus* CC398 and have concluded that transmission of LA-*S. aureus* CC398 indeed does occur between swine and humans and can result in human SSTI and BSI.[12,17,32] Apart from this study, only a single SNP-based analysis on a limited number isolates has suggested transmission of LA-*S. aureus* CC9 between humans, cattle, and swine in China[56].

The increased number of acquired AMR genes among transmission cluster isolates was largely attributable to the presence of the *tet(L)* gene conferring resistance to tetracyclines and the *vga(A)LC* gene conferring resistance to streptogramin B. This was confirmed by an increased proportion of transmission cluster isolates phenotypically resistant to tetracycline, and displaying a MDRSA phenotype, compared to non-transmission cluster isolates. While the *tet(M)* and *tet(K)* genes are commonly found in MSSA and MRSA isolates,[57] including LA-*S. aureus* CC9 and CC398,[30,58] the *tet(L)* gene is rarely detected in *S. aureus.*[57,59] The *tet(L)* gene is plasmid mediated and encodes for an efflux pump conferring resistance to tetracycline.[59] The *vga(A)LC* gene is also a plasmid mediated gene encoding for a putative efflux pump conferring resistance to

streptogramin compounds[60] and reduced susceptibility to pleuromutilins.[61] LA-MRSA CC398 carrying plasmid-borne *vga(A)* has been previously reported in IHO workers and pigs in the USA,[62] but to our knowledge, has not been reported in LA-*S. aureus* CC9. In the current study, these plasmid mediated resistance genes were enriched among transmission cluster isolates. These findings are of concern, since they may have clinical implications regarding treatment regimens for LA-*S. aureus* CC9 infections. The single SSTI-associated isolate in our study that fell into a transmission cluster carried the *vga(A)LC* gene and displayed a MDRSA phenotype.

Our study has several strengths. First, to our knowledge, our study is the first to employ core genome SNP-based analyses to examine the population structure and transmission dynamics of multidrug-resistant LA-*S. aureus* CC9 in the USA. In this study, isolates were collected from participants who lived in a region in NC with the highest density of industrial hog production in the USA,[33] and where residents and IHO workers are expressing their concerns with IHO-related exposures[63]. This study addresses this critical knowledge gap by providing evidence of transmission of multidrug-resistant LA-*S. aureus* CC9 between IHO pigs and humans in NC. We also provide evidence that this transmission does not only lead to nasal carriage but may also result in human disease, as noted by the relatedness between an SSTI-associated human isolate and an IHO pig isolate (< 20 SNP difference). Moreover, our study reveals that community residents in NC with no known exposure to livestock are also exposed to, and can carry, LA-*S. aureus* CC9 that is highly related to those collected from IHO pigs. Considering that transmission cluster isolates are largely multidrug resistant, transmission of LA-*S. aureus* CC9 between IHO-pigs and humans in the USA constitutes a major public health

concern. Second, whereas previous groups have used *spa*-type, MLST, the *scn* gene, or phenotypic resistance to tetracycline to classify *S. aureus* isolates as "livestock-associated,"[10,14,17] our study used core genome SNP-based genetic distance to classify human isolates that were closely related to IHO pig isolates. This method of classification provides a greater discriminatory capacity for identification of human isolates that are highly related to pig isolates and the associated risks of transmission of LA-*S. aureus* CC9 between IHO pigs and humans. Third, our study does not use an arbitrary SNP threshold to define transmission, as is commonly done in previous literature.[64] Rather, this analysis uses an empirical method to set a SNP threshold to define transmission clusters.

This study had several limitations. First, the international collection could be a poor representation of global LA-*S. aureus* CC9 isolates. Several SNP-based analyses have concluded intercontinental spread of particular *S. aureus* clonal groups, including LA-*S. aureus* CC398.[12,65,66] It may be the case that LA-*S. aureus* CC9 also displays intercontinental spread, but this international collection of isolates is not representative enough to capture this. However, the depth of publicly available LA-*S. aureus* CC9 sequence data is limited. Different sample processing and sequencing methods between isolates from the international and NC collection may also contribute to the observed phylogenetic relationships between LA-*S. aureus* CC9 from different countries of origin. This is likely not the case, however, considering that variability in year of collection did not explain core-genome SNP-based clustering, and contig-based AMR gene hits grouped well with core-genome SNP-based clusters. Second, our study may not fully represent IHO pig isolates from NC, USA. To study transmission between pigs and

humans, we would ideally have an equal distribution of LA-*S. aureus* CC9 collected from humans and from IHO pigs, as has been done with LA-*S. aureus* CC398[12,25]. Third, we did not have data to match IHO worker participants to the IHO where they worked, thus we are unable to explore intra- and inter-farm level transmission dynamics. This metadata is not available to us based on the request of community partners to de-couple these data to protect worker participant anonymity. We hypothesize that we would see even closer genetic relatedness between IHO worker and IHO pig LA-*S. aureus* CC9 collected from the same farm. Despite this limitation, we still found a high degree of phylogenetic relatedness between IHO pig and IHO worker LA-*S. aureus* CC9 isolates collected in NC, USA. Lastly, we are not able to provide directionality of transmission in our conclusions. This study was not aimed to reconstruct the evolutionary history of LA-*S. aureus* CC9, as has been done with LA-*S. aureus* CC398[12]. We rooted our high-resolution phylogenetic tree at the midpoint, and are, therefore, unsure if the most ancestral clade of LA-*S. aureus* CC9 is of human or animal origin. This is a research question of critical importance, and the pursuit of a current study.

SNP-based analyses have shown promise, and are now commonly used, in clinical infectious disease outbreak settings, including those concerning MRSA infection[67–69]. Our study shows that these tools can also be applied in meaningful ways within community and occupational settings to understand transmission dynamics between animals and humans in regions of the USA with intensive spatial concentration of IHOs. Industrial food animal production facilities regularly emit *S. aureus* by venting animal barns and spraying animal waste on proximal fields.[18,70] *Staphylococcus aureus* is resistant to desiccation and may persist on indoor surfaces for weeks to months.

Environmentally-disseminated LA-*S. aureus* CC9 from IHOs could contribute to contamination of household property and recreational parks,[71] for example, leading to increased exposure to multidrug-resistant pathogens and *S. aureus*-related outcomes in the community. Of particular concern was our finding that adults who reside in the community, with no known exposure to livestock, can also carry LA-MDRSA CC9 Future studies should employ WGSA to investigate environmental exposure routes implicated in transmission of LA-*S. aureus* CC9 between IHO pigs and community residents with no known exposure to livestock.

CONCLUSIONS

WGSA of LA-*S. aureus* CC9 resulted in five major findings. 1) Population structure analysis of LA-*S. aureus* CC9 revealed three distinct major lineages of LA-*S. aureus* CC9 that were associated with continent of origin. Clonal expansion of LA-*S. aureus* CC9 in NC, USA appears to be distinct from those in Europe and Asia, although similarly driven by the use of tetracycline, macrolide, and aminoglycoside antibiotics. 2) High-resolution phylogenetic analysis revealed several distinct sublineages of LA-*S. aureus* CC9, two of which included intermingled IHO pig and human isolates (transmission clusters). A high degree of phylogenetic relatedness among transmission cluster isolates strongly suggest that IHO pig and IHO human LA-*S. aureus* CC9 isolates come from a common pool. 3) Analysis of transmission cluster isolates provided evidence of transmission of LA-*S. aureus* CC9 between IHO pigs, IHO workers, IHO children, and CR adults with no known exposure to livestock, suggesting that exposure to multidrug-resistant LA-*S. aureus* CC9 is both an occupational health concern and a larger public health concern. Carriage of multidrug-resistant LA-*S. aureus* CC9 is not limited to

the occupational setting. 4) Analysis of transmission cluster isolates provided evidence that LA-*S. aureus* CC9 may result in human disease (SSTI). 5) Lastly, analysis of transmission cluster isolates suggested that LA-*S. aureus* CC9 implicated in transmission between IHO pigs and humans in NC., USA are enriched with determinants conferring resistance to multiple antibiotics that are critically important for human health.[72] Taken together, our results strongly suggest that LA-*S. aureus* CC9 from IHO pigs and humans in NC., USA come from a common pool, and that transmission between pigs and humans is associated with a multidrug-resistant phenotype and genotype. We provide the first LA-*S. aureus* CC9 reference dataset, which future studies can use to expand our understanding of LA-*S. aureus* CC9 transmission, exposure, colonization, and infection. In particular, we believe this reference dataset can be used to help in worker and community health hazard concerns regarding dynamics of LA-*S. aureus* acquisition in NC and other regions of the USA with a high density of IHOs.

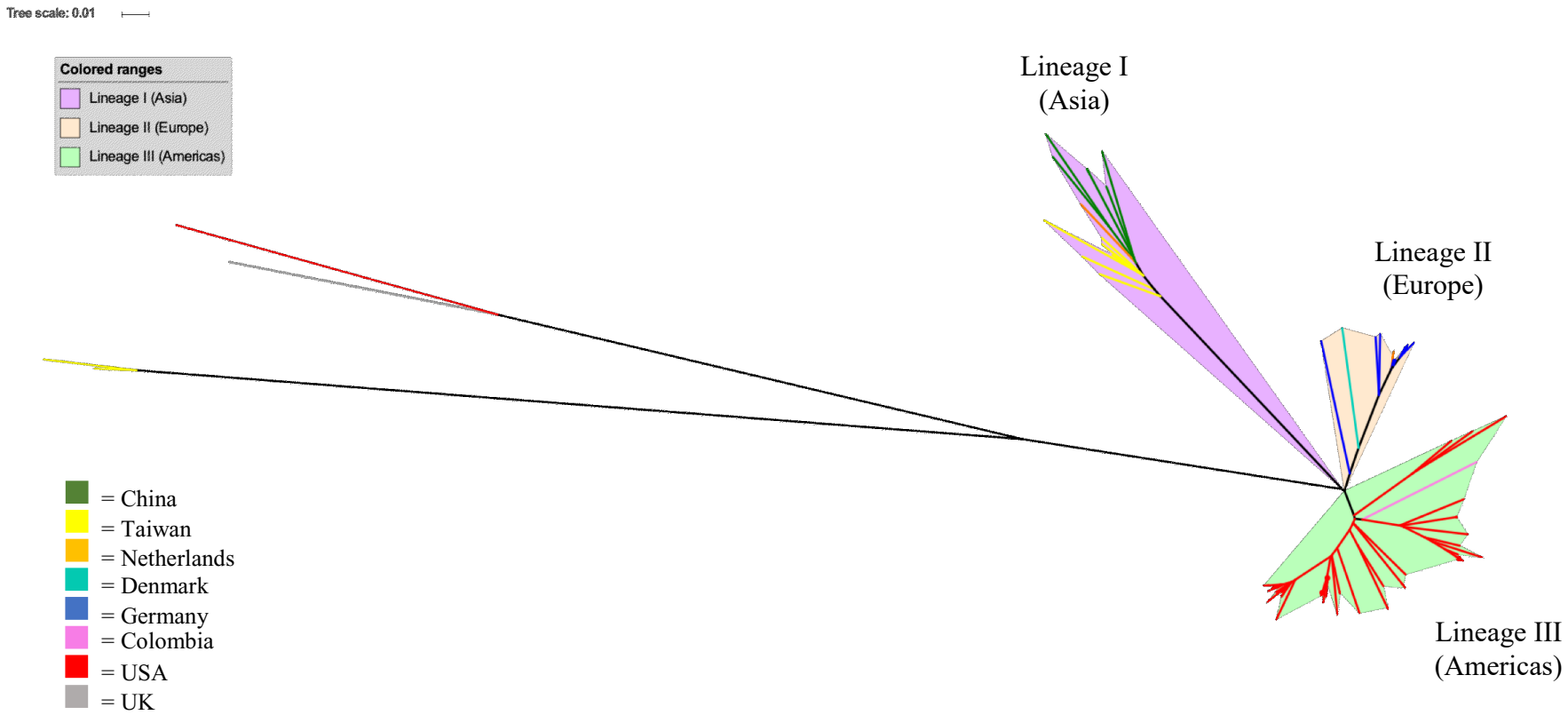
	N=50 n (%)	**Average number of acquired AMR genes/isolate** mean (standard deviation)	**Beta coefficient (95% CI)**	***p* value**
Non-transmission isolates	31 (62)	1.71 (1.24)	REF	
Putative transmission cluster isolates	19 (38)	3.84 (1.71)	2.13 (1.31, 2.95)	0.0001

Table 1.1. Comparison of the average number of acquired AMR genes between transmission cluster isolates and non-transmission cluster isolates. Beta coefficients and 95% confidence intervals were estimated using a generalized linear model, comparing the average number of acquired AMR genes/isolate among the putative transmission cluster group to the non-transmission cluster referent group.

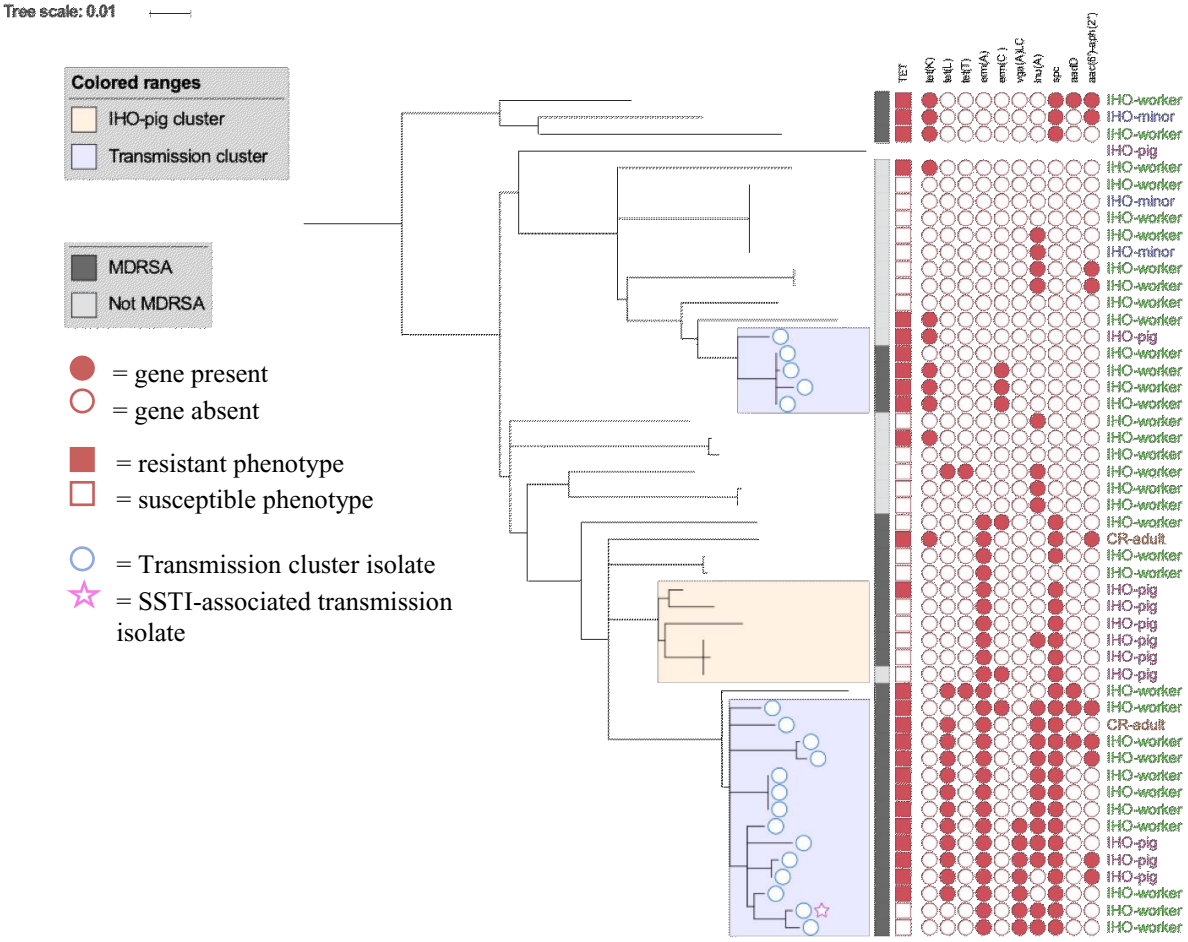
		Transmission cluster isolates N=19 n (%)	Non-transmission cluster isolates N=31 n (%)	*p* value
Tetracycline resistance	*tet(K)*	4 (21)	8 (25.8)	0.702
	tet(L)	11 (57.9)	1 (3.23)	**0.0001**
	tet(T)	0	2 (6.5)	0.258
	At least one gene	15 (79)	10 (32.3)	**0.001**
Macrolide resistance	*erm(A)*	14 (73.7)	11 (35.5)	**0.009**
	erm(C)	4 (21)	1 (3.23)	**0.041**
	At least one gene	17 (89.5)	11 (35.5)	**0.0001**
Streptogramin B resistance	*vga(A)LC*	7 (36.8)	1 (3.23)	**0.002**
Lincosamide resistance	*lnu(A)*	12 (63.2)	9 (29)	**0.018**
Aminoglycoside resistance	*aac(6')-aph(2'')*	5 (26.3)	5 (16)	0.382
	spc	14 (73.7)	13 (42)	**0.029**
	aadD	2 (10.5)	2 (6.5)	0.606
	At least one gene	14 (73.7)	9 (52.9)	0.079
Phenotypic resistance	MDRSA	18 (94.7)	13 (42)	0.0001
	Tetracycline	17 (90)	9 (29)	0.0001

Table 1.2 Antimicrobial resistance (AMR) genes and antibiotic susceptibility testing (AST) by putative transmission classification. *p values* were estimated by a Chi squared exact test. Bolded *p* values are statistically significant. *Note.* IHO = industrial hog operation; MDRSA = multidrug resistant *S. aureus*; TET = phenotypic resistance to tetracycline; transmission cluster isolates = IHO pig and human isolates separated by 42 SNP's or less.

Figure 1.1. Population structure of LA-*S. aureus* CC9. A total of 81 LA-*S. aureus* CC9 isolates from human and livestock specimens were included in this unrooted maximum-likelihood phylogeny. 32 isolates were obtained from RefSeq, and represent the international collection of LA-*S. aureus* CC9. 49 isolates were collected from IHO pigs, IHO workers, IHO children, or CR adults in North Carolina, U.S, and represent the NC collection of LA-*S. aureus* CC9. The major lineages of LA-*S. aureus* CC9 appear to correspond to continent of origin. 100% of the NC collection isolates fell into Lineage III. *Note.* IHO = industrial hog operation; CR = community referent with no known exposure to livestock.


Tree scale: 0.01
Colored ranges
Lineage I (Asia)
Lineage II (Europe)
Lineage III (Americas)
Lineage I
(Asia)
Lineage II
(Europe)
Lineage III
(Americas)
= China
= Taiwan
= Netherlands
= Denmark
= Germany
= Colombia
= USA
= UK

<u>Figure 1.2.</u> High-resolution phylogenetic analysis of Lineage III (LIII) LA-*S. aureus* CC9 isolates. 49 LA-*S. aureus* CC9 isolates collected from IHO pigs, IHO workers, IHO children, and CR adults were included in this high-resolution phylogeny. A single sublineage, denoted as the IHO pig cluster, included only IHO pig isolates and was used to set a threshold of 42 SNP's for identifying transmission clusters. Two sublineages included intermingled human and IHO pig isolates with a high degree of phylogenetic relatedness and were considered transmission clusters. Transmission cluster tended to carry a greater number of acquired antimicrobial resistance genes/isolate, and a included a greater proportion of MDRSA compared to non-transmission cluster isolates. *Note.* IHO = industrial hog operation; CR = community referent with no known exposure to livestock; MDRSA = multidrug resistant *S. aureus;* TET = phenotypic resistance to tetracycline; SSTI = skin and soft tissue infection transmission cluster isolates = IHO pig and human isolates separated by 42 SNP's or less.


Tree scale: 0.01
Colored ranges
IHO-pig cluster
Transmission cluster
MDRSA
Not MDRSA
= gene present
= gene absent
= resistant phenotype
= susceptible phenotype
= Transmission cluster isolate
= SSTI-associated transmission isolate
TET
lsa(K)
lsa(L.)
tet(T)
erm(A)
erm(C.)
vga(A).K.C
lnu(A)
spc
aadD
aac(6')-aph(2")
IHO-worker
IHO-minor
IHO-pig
CR-adult

SUPPLEMENTARY INFORMATION FOR CHAPTER 1

Table 1S.1. Isolate characteristics. Isolate characteristics are provided for 81 LA-*S. aureus* CC9 isolates included in the current study.

tree ID	Year	Country	Region	Pop struc host	host_type	Isolation source	SSTI	mdrsa	Mlst (CC)
698045	2009	China	China: Harbin	Human	Human	nares	0	.	9
698065	2009	China	China: Harbin	Human	Human	nares	0	.	9
698085	2009	China	China: Harbin	Human	Human	nares	0	.	9
1298325	2014	China	China: Shannxi	Cow	cow	milk	0	.	9
M3	2018	China	China	Pig	pig		0		9
M6	2018	China	China	Pig	pig		0		9
2250135	2014	Colombia	Colombia	Pig	pig	nares	0	.	9
638195	NA	Denmark	Denmark	Pig	pig	Bodily fluid	0	.	9
636155	NA	Germany	Germany	Pig	pig	Bodily fluid	0	.	9
636235	NA	Germany	Germany	Human	human	Bodily fluid	0	.	9
636795	NA	Germany	Germany	Chicken	chicken	Bodily fluid	0	.	9
636835	NA	Germany	Germany	Chicken	chicken	Bodily fluid	0	.	9
636935	NA	Germany	Germany	Chicken	chicken	Bodily fluid	0	.	9
637035	NA	Germany	Germany	Cow	cow	Bodily fluid	0	.	9
637075	NA	Germany	Germany	NA	.	.	0	.	9
684755	NA	Germany	Germany	NA	.	.	0	.	9
684775	NA	Germany	Germany	NA	.	.	0	.	9
684985	NA	Germany	Germany	Pig	pig	Bodily fluid	0	.	9
636515	2008	Netherlands	Netherlands	Human	human	Bodily fluid	0	.	9
636575	2010	Netherlands	Netherlands	Human	human	Bodily fluid	0	.	9
636595	2010	Netherlands	Netherlands	Human	human	Bodily fluid	0	.	9
636615	2011	Netherlands	Netherlands	Human	human	Bodily fluid	0	.	9
2247015	1998	Taiwan	Taiwan	Human	human	Inf (Wound)	1	.	9
2247065	2012	Taiwan	Taiwan	Human	human	Inf (Wound)	1	.	9
2247245	2002	Taiwan	Taiwan	Human	human	Inf (Wound)	1	.	9
2247005	2002	Taiwan	Taiwan	Human	human	Pus	0	.	9
2247045	2004	Taiwan	Taiwan	Human	human	throat	0	.	9
2247105	2010	Taiwan	Taiwan	Human	human	Synovial fluid	0	.	9
2247125	2006	Taiwan	Taiwan	Human	human	blood	0	.	9
2247315	2006	Taiwan	Taiwan	Human	human	urine	0	.	9
900042135	2006	United	United Kingdom:	NA	.	blood	0	.	9

		Kingdom	Wales						
IHOW1	2013	USA	NC	Human	IHO worker	nares	1	0	9
IHOW2	2013	USA	NC	Human	IHO worker	nares	1	1	9
IHOW3	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOC1	2013	USA	NC	Human	IHO child	nares	0	0	9
IHOW4	2013	USA	NC	Human	IHO worker	nares	0	0	9
IHOW5	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW6	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW7	2013	USA	NC	Human	IHO worker	nares	0	0	9
IHOW8	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW9	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOW10	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOC2	2014	USA	NC	Human	IHO child	nares	0	0	9
IHOW11	2014	USA	NC	Human	IHO worker	nares	0	1	9
IHOW12	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOW13	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOW14	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOC3	2014	USA	NC	Human	IHO child	nares	0	1	9
IHOW15	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOW16	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOW17	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOW18	2014	USA	NC	Human	IHO worker	nares	0	1	9
IHOW19	2014	USA	NC	Human	IHO worker	nares	0	0	9
IHOW20	2014	USA	NC	Human	IHO worker	nares	0	1	9
IHOW21	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW22	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW23	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW24	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW25	2013	USA	NC	Human	IHO worker	nares	0	0	9
IHOW26	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW27	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW28	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOW29	2013	USA	NC	Human	IHO worker	nares	0	0	9
IHOW30	2013	USA	NC	Human	IHO worker	nares	0	1	9
IHOP1	2015	USA	NC	Pig	IHO pig		0	1	9
IHOP2	2015	USA	NC	Pig	IHO pig		0	1	9

IHOP3	2015	USA	NC	Pig	IHO pig		0	0	9
IHOP4	2015	USA	NC	Pig	IHO pig		0	1	9
IHOP5	2015	USA	NC	Pig	IHO pig		0	1	9
IHOP6	2015	USA	NC	Pig	IHO pig		0	1	9
900082095	2009	USA	USA	NA	.	Inf (Wound)	1	.	9
IHOW31	2012	USA	NC	Human	IHO worker	nares	0	1	9
IHOW32	2012	USA	NC	Human	IHO worker	nares	0	0	9
IHOW33	2012	USA	NC	Human	IHO worker	nares	0	.	9
IHOW34	2014	USA	NC	Human	Work IHO	nares	0	1	9
CRA1	2014	USA	NC	Human	CR adult	nares	0	1	9
CRA2	2014	USA	NC	Human	CR adult	nares	0	1	9
IHOP7	2015	USA	NC	Pig	IHO pig	Rope	0	1	9
IHOP8	2015	USA	NC	Pig	IHO pig	Rope	0	1	9
IHOP9	2015	USA	NC	Pig	IHO pig	Rope	0	1	9
IHOP10	2015	USA	NC	Pig	IHO pig	Rope	0	0	9

	Lineage I N=13	Lineage II N=14	Lineage III N=50	All other isolates N=4	Total N=81
Host	n (%)	n (%)	n (%)	0	n (%)
Human	10 (77)	4 (29)	39 (78)	2 (50)	55 (68)
Pig	2 (15)	3 (21)	11 (22)	0	16 (20)
Chicken	0	3 (21)	0	0	3 (4)
Cow	1 (8)	1 (7)	0	0	2 (2)
NA	0	3 (21)	0	2 (50)	5 (6)
Country of origin					
USA	0	0	49 (98)	1 (25)	50 (62)
Colombia	0	0	1 (2)	0	1 (1)
Germany	0	10 (71)	0	0	10 (12)
Netherlands	1 (8)	3 (21)	0	0	4 (5)
Denmark	0	1 (7)	0	0	1 (1)
China	6 (46)	0	0	0	6 (7)
Taiwan	6 (46)	0	0	2 (50)	8 (10)
United Kingdom	0	0	0	1 (25)	1 (1)
Collection					
NC	0	0	49 (98)	0	49 (60)
International	13 (100)	14 (100)	1 (2)	4 (100)	32 (40)

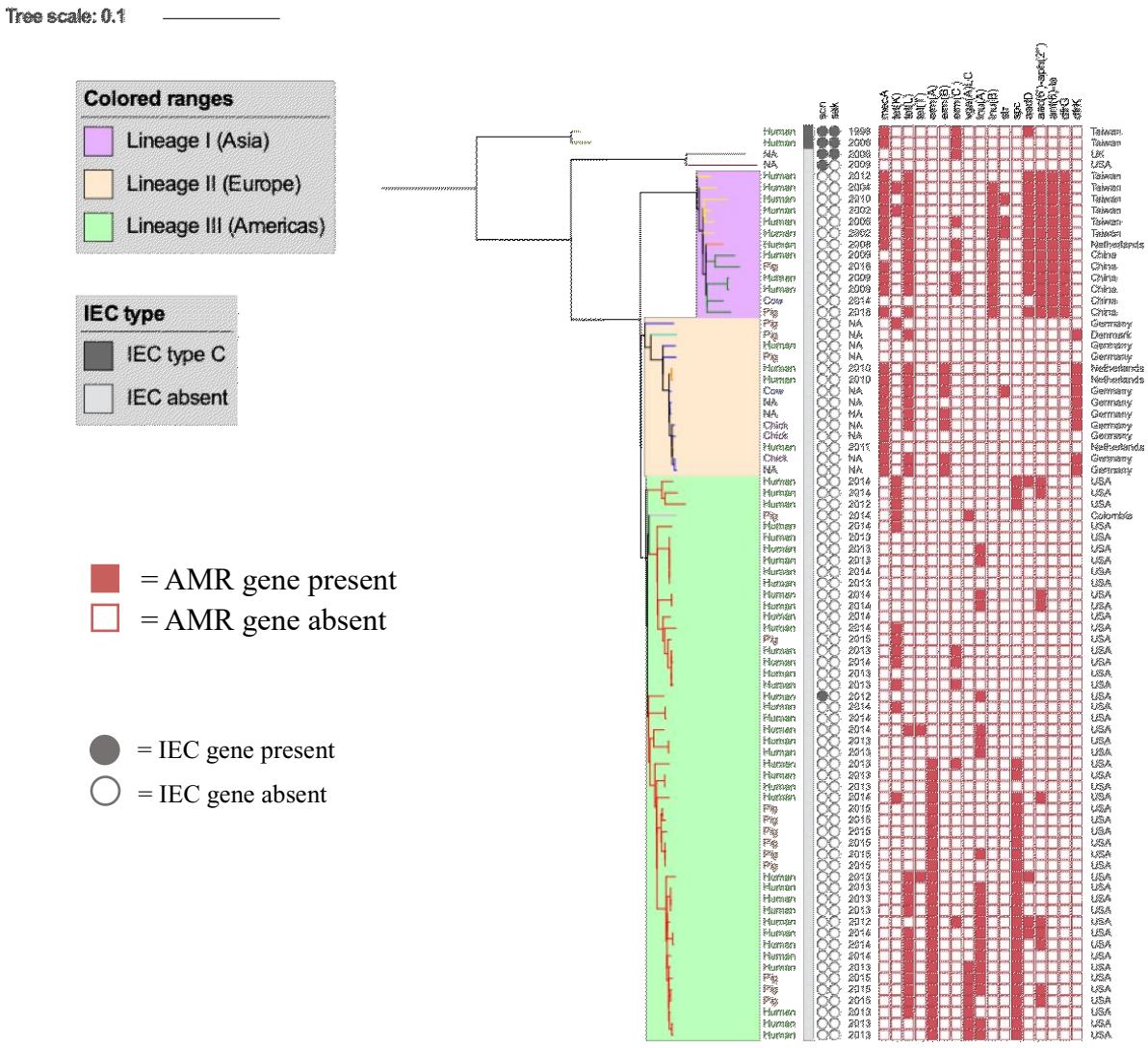
Table 1S.2. Epidemiological characteristics of isolates by major lineage of LA-*S. aureus* CC9.

Table 1S.3. Presence of Immune evasion cluster (IEC) and antimicrobial resistance (AMR) genes by major lineage of LA-*S. aureus* CC9. *p values* were estimated by a Chi squared exact test. Bolded *p* values are statistically significant.

		Lineage I	**Lineage II**	**Lineage III**		
		N=13	**N=14**	**N=50**	*p value*	*p value*
		n (%)	n (%)	n (%)	(LIII vs. LI)	(LIII vs. LII)
Immune Evasion Cluster (IEC) genes	*scn*	0	0	1 (2)	NA	NA
	sak	0	0	0	NA	NA
Methicillin resistance	*mecA*	11 (84.6)	10 (71.4)	0	**0**	**0**
Tetracycline resistance	*tet(K)*	2 (15.4)	1 (7.1)	12 (24)	0.506	0.166
	tet(L)	11 (84.6)	9 (64.3)	12 (24)	**0**	**0.005**
	tet(T)	0	0	2	0.464	0.447
	At least one gene	11 (84.6)	10 (71.4)	25 (50)	**0.025**	0.155
Macrolide resistance	*erm(A)*	0	0	25 (50)	**0.001**	**0.001**
	erm(B)	0	7 (50)	0	NA	**0.0001**
	erm(C)	6 (46.2)	0	5 (10)	**0.002**	0.218
	At least one gene	6 (46.2)	7 (50)	28 (56)	0.526	0.69
Streptogramin B resistance	*vga(A)LC*	0	0	8 (16)	0.123	0.11
Lincosamide resistance	*lnu(A)*	0	0	21 (42)	**0.004**	**0.003**
	lnu(B)	11 (84.6)	0	0	**0**	NA
	At least one gene	11 (84.6)	0	21 (42)	**0.004**	**0.003**
Aminoglycoside resistance	*str*	2 (15.4)	1 (7.1)	0	**0.005**	0.057
	ant(6)-la	12 (92.3)	0	0	**0**	NA
	aac(6')-aph(2'')	12 (92.3)	0	10 (20)	**0**	0.069
	spc	0	0	27 (54)	**0**	**0**
	aadD	11 (84.6)	0	4 (8)	**0**	0.274
	At least one gene	12 (92.3)	1 (7.14)	31 (62)	**0.021**	**0.001**
Trimethoprim resistance	dfrG	12	0	0	**0**	NA
	dfrK	0	9	0	NA	**0**
	At least one gene	12 (92.3)	9 (64.3)	0	**0**	**0**

	Transmission cluster isolates	**All other isolates**	**Total**
	N=19	**N=31**	**N=50**
	n (%)	n (%)	n (%)
Host			
IHO-pig	4 (21)	6 (19)	10 (20)
IHO-worker	14 (73.7)	21 (68)	35 (70)
IHO-minor	0	3 (10)	3 (6)
CR-adult	1 (5.3)	1 (3)	2 (4)

Table 1S.4. Epidemiological characteristics of LA-*S. aureus* CC9 isolates by transmission cluster classification. *Note.* IHO=industrial hog operation; transmission cluster=IHO pig and human isolates separated by less than 42 SNP's.



<u>Figure 1S.1.</u> Maximum likelihood phylogeny of 49 LA-*S. aureus* CC9 isolates from the NC collection and 32 LA-*S. aureus* CC9 isolates from the international collection with detailed genotyping and epidemiological data. Note. IEC = Immune evasion cluster; AMR = antimicrobial resistance; Chick = chicken.

CHAPTER 2

Comparison of livestock-associated and community-associated *Staphylococcus aureus* pathogenicity in a mouse model of skin and soft tissue infection

ABSTRACT

Introduction. Industrial hog operation (IHO) workers are at increased risk of carrying *Staphylococcus aureus* in their nares, particularly strains that are livestock-associated (LA) and multidrug-resistant. The pathogenicity of LA-*S. aureus* strains remains unclear, with some prior studies suggesting reduced transmission and virulence in humans compared to community-associated methicillin-resistant (CA-MRSA) *S. aureus*.

Objectives. The objective of this study was to determine the degree to which LA-*S. aureus* strains contracted by IHO workers cause disease relative to a representative CA-MRSA strain in a mouse model of skin and soft tissue infection (SSTI).

Results. Mice infected with CC398 LA-*S. aureus* strains (IHW398-1 and IHW398-2) developed larger lesion sizes with higher bacterial burden than mice infected with CA-MRSA (SF8300) ($p < 0.05$). The greatest lesion size and bacterial burden was seen with a CC398 strain that produced a recurrent SSTI in an IHO worker. The LA-*S. aureus* infected mice had decreased IL-1β protein levels compared with CA-MRSA-infected mice ($p < 0.05$), suggesting a suboptimal host response to LA-*S. aureus* SSTIs. WGSA revealed heterogeneity in virulence factor and antimicrobial resistance genes carried by LA-*S. aureus* and CA-MRSA strains.

Conclusions. The observed pathogenicity suggest LA-*S. aureus* are highly pathogenic in a mouse model of SSTI and that more attention should be placed on preventing the spread of LA-*S. aureus* into human populations.

INTRODUCTION

In the past decade it has become evident that animal-adapted multidrug resistant *Staphylococcus aureus* (MDRSA) has emerged among food animals raised in concentrated animal feeding operations (CAFOs) and individuals who have frequent contact with food animals raised in CAFOs globally,[46,73] including the United States.[14,17,23] A study conducted in Pennsylvania concluded that residential proximity to swine CAFO manure land application crop fields was associated with increased odds of methicillin-resistant *S. aureus* (MRSA) infection and skin and soft tissue infection (SSTI),[11] suggesting an environmental exposure pathway wherein community members could become infected with antimicrobial-resistant *S. aureus* originating at swine CAFOs. This is consistent with evidence suggesting that industrial hog operation (IHO) workers are at an increased risk of carrying livestock-associated (LA) *S. aureus*, including LA-MDRSA, intranasally.[9,14–16] LA-MDRSA originating at IHOs may also be carried and transmitted from IHO workers to family contacts, noted by an increased prevalence of MRSA and MDRSA nasal carriage among children living with IHO workers in North Carolina compared to children living with community resident adults with no livestock exposure.[10] Less is known about the extent to which nasal carriage of such LA-*S. aureus* is associated with infection, particularly skin and soft tissue infections (SSTIs).

While large-scale surveillance studies from Europe suggest that LA-MRSA strains are capable of causing the full suite of human infections,[25] some reports suggest that these strains display a decreased capacity for human-to-human transmission and may be less pathogenic than typical community associated (CA)- and hospital associated

(HA)-*S. aureus* strains.[12,19–22] USA300 is a hypervirulent clone of CA-*S. aureus* that emerged in the USA in the late 1990's, and has become the dominant CA-MRSA strain circulating in North America.[66] Consistent with its ability to cause severe and widespread disease, USA300 clone, SF8300, displayed considerably increased virulence in a mouse model of skin and soft tissue infection (SSTI) compared to other MRSA lineages.[27]

At a genetic level, LA-*S. aureus* tend to lack genetic factors associated with human infection that are typically found in CA-MRSA lineages, including the human immune evasion cluster (IEC) genes (*scn*) and Panton-Valentine leukocidin (PVL)-encoding genes (*lukS-PV* and *lukF-PV*) .[12,74,75] Nevertheless, LA-*S. aureus* strains have been reported to produce skin and bloodstream infections in humans in Europe,[13,25,26,76,77] the USA,[15,16,32,78,79] and Canada.[24] A recent study concluded that a LA-MDRSA isolate collected from poultry displayed greater lethality in a murine sepsis model compared to a clinical methicillin-susceptible *S. aureus* (MSSA) isolate and provided information on differential gene expression.[39] To our knowledge, no studies have assessed the relative pathogenesis of LA-*S. aureus* acquired from swine on an IHO compared to a well-characterized and highly pathogenic CA-MRSA strain isolated from a human SSTI outbreak in the community. Considering that LA-*S. aureus* has surfaced in community members, including children, who experience environmental exposure to CAFOs, it is critical to improve our understanding of the pathogenic potential of LA-*S. aureus* strains emerging from CAFO environments and resulting in human SSTI. This study aims to understand the degree to which LA-*S. aureus* strains contracted by IHO workers cause

disease relative to a representative hypervirulent CA-MRSA strain -- *i.e.*, USA300 clone, SF8300,[37,38] in a mouse model of SSTI.

METHODS

Selection of S. aureus *isolates.*

One CC9 and one CC8 LA-MDRSA strain were selected that were collected from the anterior nares of two IHO workers who reported a SSTI within the past 3 months between October 2013 and February 2014 in a prospective cohort study of IHO workers in North Carolina (NCHW9 and NCHW8).[15] Two CC398 LA-MDRSA isolates were selected that were collected directly from two IHO workers' active skin infections between May 2011 and February 2013 in a prospective cohort study of IHO workers in Iowa,[16] and were provided by Dr. Tara C. Smith from Kent State University (IHW398-1 and IHW398-2). IHW398-1 was responsible for a physician-diagnosed recurrent SSTI in a male hog worker.[32] The representative CA-MRSA strain SF8300 was isolated from a SSTI in a patient treated at the San Francisco General Hospital and was provided by Dr. Henry Chambers of University of California San Francisco. SF8300 is pulsed-field type USA300, which is the type responsible for the vast majority of CA-MRSA SSTI in the USA, Canada, and Europe,[37] and displays a high degree of pathogenicity in a mouse model of SSTI.[38] Hemolytic activity was treated as a binary variable and was confirmed for each isolate by observation of a zone of hemolysis after 24-hours of growth on blood sheep agar plates.

Indicators of livestock association.

There is currently no established molecular marker for LA-*S. aureus.* LA-MDRSA has been consistently classified as: (i) MDRSA carriage or infection in humans arising from exposure to livestock, (ii) belonging to the clonal complex 398 (CC398) or CC9, and (iii) lacking the staphylococcal complement inhibitor gene *scn* (*scn-)*.[14,15] Here we used CC9 or CC398 within the IIa livestock clade of the CC398 phylogeny, and the absence of the *scn* gene (*scn*-) as indicators of LA-*S. aureus*. Putative clonal complex (CC) was assigned to each isolate based on *spa type*, using the Ridom StaphType software and the Ridom SpaServer (http://spa.ridom.de/index.shtml).

Antimicrobial susceptibility testing.

Each selected *S. aureus* strain was subjected to a panel of antibiotics for antibiotic susceptibility testing (AST) using the Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD) by the the Clinical Microbiology Laboratory at the Johns Hopkins Hospital, according to guidelines for clinical isolates. MRSA was defined as resistant to cefoxitin or oxacillin and positive for the *mecA* or *mecC* gene. MDRSA was defined as resistant to greater than or equal to three classes of antibiotics. MIC cut off's used to establish resistant, intermediate, or susceptible phenotypes, and antibiotic abbreviations, are provided in Chapter 3: Supplementary Information (Table 3S.1).

S. aureus *growth curves.*

S. aureus strains were streaked onto tryptic soy agar plates and grown overnight. Single colonies were selected and grown in tryptic soy broth (TSB) at 37°C in a shaking incubator overnight, shaking at 240 rpm and then sub-cultured at a 1:50 dilution in TSB. At 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 hours, 100 µL of the sub-cultures were pipetted

onto a 96-well plate and absorbance (600 nm) was read with a Synergy H1 Hybrid Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

Mice.

Mice with a C57BL/6 genetic background were used in all experiments. Mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained under specific-pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at Johns Hopkins and housed according to procedures described in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011).

Mouse model of* S. aureus *skin and soft tissue infection.

Animal care and all experiments were approved and performed in accordance with the guidelines and regulations approved by the Johns Hopkins University Animal Care and Use Committee, which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (8th edition, 2011). As a first step toward identifying the relative pathogenicity of LA-*S. aureus* compared to that of CA-MRSA, we evaluated the skin lesions that developed in response to intradermal (i.d.) infection of four LA-MDRSA isolates collected from IHO workers and a representative CA-MRSA strain (SF8300) in a mouse model of SSTI (Figure 2.2A). The SF8300 strain was chosen as a representative CA-MRSA strain for this study because it is the same pulsed-field USA300 type responsible for the epidemic of CA-MRSA SSTI in humans in the USA[37] and has been previously used to compare

pathogenicity of *S. aureus* cutaneous infections in mice.[38] The upper backs of C57BL/6 mice were shaved and inoculated intradermally with 3×10^7 colony-forming units (CFU) in 100 µl PBS of midlogarithmic growth phase SF8300 (CC8) (n=20 mice) or the following LA-*S. aureus* strains (n=10 mice/strain) BP772 (CC8), CA746 (CC9), 15606P (CC398), or 172784P (CC398). Digital photographs of mice taken on days 0, 1, 3, 7, 10, and 14 post-challenge were analyzed for measurements of total lesion size (area, cm^2) using ImageJ software. Data reported as mean total lesion size (cm^2) ± the standard error of the mean (SEM).

Measuring bacterial burden.

Mice (n=5/group) were euthanized on day 3 post-infection and 10-mm skin punch biopsies of lesions, taken from the middle of the lesion, were homogenized (Pro200 Series homogenizer; Pro Scientific, Oxford, CT) in PBS on ice. Samples were serially-diluted and cultured on TSA plates overnight at 37°C and CFU were enumerated.

Cytokine, chemokine, and growth factor evaluation in infected skin.

Infected skin biopsies from day 3 were analyzed for protein levels of cytokines, chemokines and growth factors to provide insights into the host response to CA-MRSA or LA- *S. aureus* i.d. inoculation. Mice (n=5/group) were euthanized on day 3 post-infection and 10-mm skin punch biopsies of lesions were weighed and snap-frozen in liquid nitrogen. On ice, each specimen was homogenized with a hand-held homogenizer (Pro200 Series homogenizer; Pro Scientific) in Protein Lysis Buffer (Promega) containing protease inhibitor cocktail (Roche). All samples were stored at 80°C. Samples were then centrifuged at 4°C and supernatants were assayed for total protein, and protein

levels of cytokines, chemokines and growth factors using a 9-plex and 11-plex mouse protein array, according to the manufacturer's recommendations (Bio-Plex Pro™, Biorad; Hercules, CA). Total protein concentration was determined using NanoDrop. For the 9-plex and 11-plex arrays, samples were normalized to 0.75 mg/mL and 2 mg/mL total protein, respectively. Samples were also assayed for myeloperoxidase (MPO) levels using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

Genome sequencing.

We sequenced and assembled the genomes of the LA-MDRSA isolates, and used the publicly available genomic sequence of SF8300[37] for WGSA. DNA was prepared for multiplexed, paired-end sequencing on an Illumina MiSeq (Illumina, Inc., San Diego, CA). For each isolate, 500 ng of DNA was sheared to an average fragment size of 300 bp in 50 µL using a Covaris E220 Focused-Ultrasonicator (Covaris, Woburn, MA). End repair, A-tailing, and adaptor ligation of the total volume of sheared DNA was performed using the Kapa Hyper Prep Kit (Kapa Biosystems, Inc., Wilmington, MA) with recommended adaptor concentrations for libraries constructed from 500 ng input DNA. Uniquely barcoded adaptors used in adaptor ligation were obtained from BioO Scientific® (BioO Scientific®, Austin, TX, NEB, NEXTflex-96™ DNA Barcodes for DNA). Following ligation of adaptors, KAPA Pure Beads (Kapa Biosystems, Inc., Wilmington, MA) were used to perform a 0.8X bead-based cleanup for each DNA library. Individual libraries were quantified in triplicate at two concentrations (1:100 and 1:1000) via quantitative PCR using the Kapa Library Quantification kit (Kapa Biosystems, Inc. Wilmington, MA,). Based on individual library concentrations, equimolar pools of *S. aureus* libraries were prepared at a concentration of at least 1 nM.

The pooled libraries were quality controlled on an Agilent bioanalyzer and sequenced on an Illumina MiSeq at 2 × 300 bp.

De novo assembly and molecular characterization of S. aureus genomes.

To gain information on the genetic basis for the pathogenic potential of LA-MDRSA as compared to SF8300, we assessed the presence of core and mobile genetic element (MGE) encoded virulence factors (VF's) and acquired antimicrobial resistance (AMR) genes important for pathogenesis in humans. Illumina short-read sequences were trimmed using trimmomatic[80], and assembled into contigs using the SPADES assembler (v.3.5).[81] Assembly quality was assessed using QUAST (v.2.3),[82] and reference MLST housekeeping genes were identified using BLAST (Version 2.2.25+)[83] at 100% query coverage and nucleotide identity (ID). An in-house script was used to match genes and MLST profile data[84] to determine the final MLST type. These analyses were performed on the GWU Colonial High Performance Computing Cluster. Virulence factor (VF) and Antimicrobial resistance (AMR) genes were determined by uploading each assembled *S. aureus* genome onto the *S. aureus* VirulenceFinder 1.5[53] and ResFinder 3.0,[54] available on the Center for Genomic Epidemiology (CGE) server. ABRicate (https://github.com/tseemann/abricate), a modified BLASTn based tool for the screening of genes in assemblies, was used with a custom database containing microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and several additional hemolysins and leucocidins, to detect the following genes on the assembled *S. aureus* genomes: *clfA* (GenBank accession no. Z18852) and *clfB* (GenBank accession no. AJ224764) encoding clumping factor A and B (ClfA and ClfB), *sdrC* (GenBank accession no. AJ005645), *sdrD* (GenBank accession no. AJ005646), and *sdrE* (GenBank

accession no. AJ005647) encoding serine-aspartate repeat protein C, D, and E (SdrC, SdrD, and SdrE), *bbp* (GenBank accession no. BX571856) encoding bone sialoprotein-binding protein (Bbp), *fnbpA* (GenBank accession no. J04151) and *fnbpB* (GenBank accession no. X62992) encoding Fibrinogen-binding protein A and B (FnBPA and FnBPB), and *cna* (GenBank accession no. M81736) encoding collagen adhesin (Cna), *hla* (GenBank accession no. CP000255 , BX571856, BX571857, BA000017, BA000033, BA000018, CP000046) encoding for alpha hemolysin, *hlb* (GenBank accession no. BX571856, BX571857, BA000017, BA000033, BA000018, CP000046) encoding for beta hemolysin, *lukA* and *lukB* (GenBank accession no. AP009351) encoding for the LukAB leucocidin, and *nor* (GenBank accession no. BX571856) encoding for nitric oxide reductase. For all genome hits, the threshold of ID was set to 85% and percentage of minimum gene length was set to 60%. All genome hits were manually inspected for confirmation.

Statistical analysis.

Data were compared using Student's *t* test (two-tailed), comparing each LA-*S. aureus* strain to the representative SF8300 CA-MRSA referent strain. p-values < 0.05 were considered statistically significantly.

RESULTS

***LA*-S. aureus *and antibiotic susceptibility testing*.**

Of the 17 antibiotics tested, complete or intermediate resistance was observed to all antibiotics except for vancomycin, linezolid, daptomycin, rifampicin, and

nitrofurantoin (Figure 2.1). All *S. aureus* clones were resistant to ampicillin and penicillin, and displayed a multidrug resistant phenotype, while only the CA-MRSA clone SF8300 displayed a methicillin-resistant phenotype. The SF8300 isolate also displayed complete resistance to erythromycin and cefotaxime, and intermediate resistance to quinupristin/dalfopristin. The NCHW8 isolate, collected from an IHO worker's nares, additionally displayed resistance to erythromycin, clindamycin, and gentamycin. Both CC398 isolates displayed resistance to tetracycline and intermediate resistance to quinupristin/dalfopristin and minocycline, while the IHW398-1 isolate, responsible for a recurrent SSTI in an IHO worker, also displayed resistance to moxifloxacin and sulfamethoxazole/trimethoprim. The SSTI-associated CC9 isolate displayed resistance to erythromycin, clindamycin, and moxifloxacin.

LA-S. aureus-*infected mice and skin lesion size.*

Growth curves showed a mid-logarithmic phase at 3 hours for all isolates, and there were no statistically significant differences in growth curves between isolates (Figure 2M.1). Mice were inoculated i.d. with $3x10^7$ CFU's of mid-logarithmic growth phase *S. aureus* and skin lesion sizes were evaluated over time (Figure 2.2B). All of the *S. aureus* strains resulted in visible skin lesions that healed by day 14. SF8300 and NCHW9 infected mice developed maximum lesion size on day 7 of 0.9 ± 0.1 cm^2 and 0.8 ± 0.1 cm^2, respectively. IHW398-1, IHW398-2, and NCHW8 infected mice developed maximum lesion sizes on day 3 of 1.3 ± 0.1 cm^2, 1.0 ± 0.1 cm^2, and 0.9 ± 0.2 cm^2, respectively. Both CC398 LA-MDRSA strains collected directly from IHO workers with

active SSTI developed significantly larger lesion sizes on day 3 compared with SF8300 ($P < 0.05$) (Figure 2.2B). The skin lesions sizes of all other LA-MDRSA isolates (CC9 or CC8) were not statistically different from those of SF8300 at any time point. Of note, mice infected with the IHW398-1 clone which was responsible for a recurrent SSTI in an IHO worker, developed the largest lesion sizes, peaking on day 3 and were 2-fold greater than those of SF8300-infected mice (Figure 2.2C).

LA*-S. aureus-*infected mice and bacterial burden.

Given that the CC398 lesion sizes peaked on day 3, we evaluated the bacterial burden by determining *ex vivo* CFU isolated from skin punch biopsy specimens of the entire skin lesions obtained on day 3. *Ex vivo* CFU from CC398 (IHW398-1 and IHW398-2) and CC9 LA-MDRSA-infected mice were significantly greater compared with SF8300-infected mice (Figure 2.3), suggesting increased bacterial proliferation and/or persistence of these particular LA-MDRSA strains *in vivo*.

LA*-S. aureus-*infected mice and local cytokine, chemokine, and growth factor levels.

At day 3 post-infection, SF8300-infected mice had significantly greater protein levels of IL-1β compared with all LA-MDRSA-infected mice ($P < 0.05$) (Figure 2.4A). IHW398-1-, IHW398-2-, and NCHW9-infected mice had significantly greater protein levels of IL-6 compared with the CC8 strains SF8300 and BP772 ($P < 0.05$). IHW398-1-, IHW398-2-, and NCHW9-infected mice also had significantly increased protein levels IL-12(p70) compared with SF8300-infected mice ($P < 0.05$). Proteins that showed no difference between LA- and CA- *S. aureus* infected mice were IL-10 (Figure 2.4A), TNF, IL-12(p40), LIF, MIG, M-CSF, VEGF, and MPO and several cytokines were below the

limit of detection (IFN-γ, IL-4, IL-5, and IL-17A) (Figure 2.4A and B). With respect to chemokines, the neutrophil-attracting chemokine MIP-2 were significantly greater in day 3 lesions of SF8300 infected mice compared to lesions of NCHW8, IHW398-1, and NCHW9 infected mice ($P < 0.05$) (Figure 2.4A). Taken together, LA-*S. aureus* strains had lower IL-1β and MIP-2 but higher levels of IL-6 and IL-12(p70) compared with SF8300.

Whole-genome sequencing analysis (WGSA) of CA- and LA-S. aureus.

Figure 2.5 displays genomes hits for VFs and acquired AMR genes present in at least one of the five *S. aureus* genomes used in this study. WGSA of VF genes led to the following key findings (Figure 2.5A). First, MSCRAMM genes *clfA, clfB, fnbA, and fnbB,* known to play a role host cell adhesion,[85] are present among CA- and LA- *S. aureus* strains. Second, human IEC genes *scn* and *sak* were present among CC8 isolates and were absent from LA-MDRSA CC398 and CC9 isolates. Third, the *splA, splB, and splC* genes encoding serine proteases, and reported to increase virulence *in-vivo,* present only among the CC8 strains.[86] The *nor* gene encoding for a nitric oxide reductase enzyme has also been reported to increase *S. aureus* virulence but was only present among the LA-*S. aureus* CC398 strains.[87] Fourth, hemolysin genes *hla,* known to play a critical role in dermo-necrosis,[88–91] *and the hlgA, hlgB, and hlgC* genes were conserved across CA-MRSA and LA-*S. aureus* isolates. *hlb* appeared to be truncated in the CA-MRSA strain and intact in the LA-*S. aureus* strains. Fifth, the leukotoxin encoding genes *lukD* and *lukE,* known to induce inflammation and dermonecrosis,[92,93] were present among CC8 *S.*

aureus isolates, but only the SF8300 CC8 isolate carried the Panton-Valentine Leukocidin encoding genes *lukF-PV* and *lukS-PV*. *lukA* and *lukB* genes encoding for the bicomponent LukAB leucocidin were present among CA- and LA- *S. aureus* genomes. Lastly, neither CC398 LA-MDRSA isolates contained any known enterotoxin genes typically encoded on prophages and pathogenicity islands. The genes encoding enterotoxin K (*sek)* and enterotoxin Q (*seq)* were conserved across CC8 isolates, while the SSTI-associated NCHW8 CC8 isolate additionally carried the enterotoxin A (*sea)* and enterotoxin C (*sec)* encoding genes. Unique to the CC9 LA-MDRSA isolate was the egc cluster, encoding for enterotoxin G, I, M, N, and O (*seg, sei, sem sen, seo*)..

WGSA of AMR genes led to the following key findings (Figure 2.5B). First, the *mecA* gene, encoding for methicillin resistance, was absent from LA-MDRSA, but these isolates carried the beta-lactamase encoding gene *blaZ*. Second, both CC398 and the CC9 LA-MDRSA genomes encoded for tetracycline resistance. The CC398 isolates carried a *tet(M)* gene, while the CC9 isolate carried a *tet(L)* gene. Third, macrolide resistance encoding genes were found only in LA-MDRSA isolates. The NCHW8 and NCHW9 isolate carried the erythromycin resistance encoding gene *erm(A)*, and the IHW398-2 and NCHW9 isolates carried the ATP-binding protein encoding gene *vga(A)* conferring resistance to streptogramin A antibiotics. Fourth, unique to the IHW398-1 isolate, responsible for a recurrent SSTI in an IHO worker, was the *dfrG* gene encoding resistance to trimethoprim antibiotics. Fifth, the *spc* gene, conferring resistance to spectinomycin, was found only in the LA-MDRSA isolates collected from IHO workers in North Carolina (NCHW8 and NCHW9). Finally, unique to the NCHW8 isolate was the *aac(6')-aph(2")* gene conferring resistance to gentamycin.

DISCUSSION

The results of this study demonstrate an increased degree of pathogenicity associated with LA-*S. aureus*, marked by increased lesion size and bacterial burden, compared to CA-MRSA in a mouse model of SSTI. The IHW398-1 isolate in particular, which belongs to the LA-CC398-IIa lineage,[32] displayed the greatest degree of pathogenicity and was a LA-MDRSA isolate. It is notable that the lesion size of the NCHW9 LA-CC9 isolate was equivalent to the CA-MRSA strain (SF8300). Additionally, the *S. aureus* CFU counts recovered from the NCHW9 LA-CC9 infected lesions were significantly greater than the CA-MRSA strain (SF8300) and comparable to those of the highly pathogenic IHW398-1 LA-CC398-IIa strain. The CC9 LA-MDRSA strain appears to display increased bacterial proliferation and/or persistence *in vivo*. This is important because the CC9 lineage of *S. aureus* is emerging as a predominant clone among IHO workers and community residents in North Carolina[10,15] It is critical to continue to monitor the spread of LA-*S. aureus* CC9 into human populations, and further elucidate mechanisms of virulence and persistence. In certain regions of the world , LA-*S. aureus* has successfully disseminated into the human community and is a major contributor to human morbidity, accounting for 25% of MRSA isolated in parts of Europe.[13] IHO workers are at an increased risk of carrying LA-MDRSA,[9,15,45] and developing LA-*S. aureus* SSTI.[11,15,16] As our efforts to understand and combat the environmental and community origins of antimicrobial resistant *S. aureus* infections become a priority in the USA, it is critical to examine the pathogenic potential of LA-*S. aureus* that have contributed to human morbidity.

We also observed that the host cytokine and chemokine response to LA-MDRSA strains differed from that of CA-MRSA. Neutrophil derived IL-1β plays a critical role in amplifying and maintaining a neutrophilic response for abscess formation and bacterial clearance,[94] and previous studies have reported that *S. aureus*-infected IL-1β-deficient mice develop larger skin lesions with higher bacterial counts than wildtype mice.[95] An optimal response to *S. aureus* SSTI is associated with elevated local IL-1β protein levels[89,94–97]. Furthermore, engulfment of *S. aureus* by polymorphonuclear cells (PMNs) is known to alter macrophage production of IL-6, which subsequently lowers secretion of IL-1β.[96] Our cytokine results indicated that the host response to LA-*S. aureus* infections might be suboptimal, as LA-*S. aureus*-infected mice had larger lesions, higher bacterial burden and decreased IL-1β and MIP2 and increased IL-6 levels compared with SF8300-infected mice. A possible explanation for these findings is that LA-*S. aureus* have a distinct VF repertoire that might have suppressed the optimal IL-1β neutrophilic response required for bacterial clearance of CA-MRSA from the site of the SSTI. *S. aureus* alpha-toxin, for example, has been shown to suppress local IL-1β production in a mouse model of SSTI.[89]

Although all of the isolates displayed phenotypic hemolysis, our WGSA revealed that only LA-*S. aureus* CC398 and CC9 carried an intact beta-hemolysin gene. An insertion of the Sa3 prophage into the beta-hemolysin gene[55] results in a truncated beta-hemolysin gene in both of the CC8 genomes (SF8300 and NCHW8). A previous study has reported that mutant strains lacking the Sa3 prophage, thereby carrying an intact beta-hemolysin gene, display a greater degree of hemolysis compared to its wild type.[98] An intact *hlb* gene has been previously shown to increase *S. aureus* fitness for colonization

and persistence in mice.[98] However, a critical role for *hlb* in the pathogenesis of SSTI is not entirely clear.[88] The gain of an intact beta-hemolysin gene is dependent on the loss of human immune evasion cluster (IEC) genes – the Sa3 prophage can carry several human IEC genes including *scn* and *sak,*[55] which were absent from the genomes of the LA-*S. aureus* CC9 and CC398 isolates (NCHW9, IHW398-1, and IHW398-2). The staphylococcal complement inhibitor protein has been reported to be highly immunogenic[99] and functional only against components of the human immune system.[55] For these reasons, it may be the loss of the Sa3 prophage and its associated proteins that provides improved virulence for LA-*S. aureus* CC398 in a mouse model of SSTI. Furthermore, the *nor* gene, commonly associated with pathogenic bacteria and hypothesized to provide improved virulence and fitness for *S. aureus* infections *in vivo*,[87] was also only found among the LA-*S. aureus* CC398 isolates that produced the largest lesions (IHW398-1 and IHW398-2). Taken together, there are many genetic differences between *S. aureus* CC8 and CC398 that could be responsible for the observed lesion sizes, including the gain or loss of genes encoding for important hemolytic toxins or immune evasion proteins.

Our study had several strengths. First, to our knowledge, this was the first experimental design involving LA-*S. aureus* strains isolated from nasal carriage and SSTI events of IHO workers in the United States. Second, this study was the first, to our knowledge, to characterize the relative pathogenicity of a LA-*S. aureus* CC9 strain, which appears to be an emerging clone among IHO workers in North Carolina and Asia.[10,15,30] Finally, we compared all LA-*S. aureus* strains to a well-characterized USA300 CC8 strain that has consistently demonstrated substantial pathogenicity in

C57BL/6 mouse models of SSTI and capacity to cause human infections in community settings.[37,38]

Our study also has several limitations. First, certain *S. aureus* virulence factors (VFs) expected to increase pathogenicity may have low, or no activity in a mouse model of SSTI, such as PVL, HlgAB, and HlgBC.[92] Furthermore, LA-*S. aureus* largely do not carry human immune evasion cluster (IEC) genes, *scn* and *sak*[55], suggesting a host adaptation to animals.[12] Thus, these results should be interpreted within the limitations of using a mouse model to compare the virulence of the included *S. aureus* strains. Second, this study was done with a single large dose inoculum. Future studies should conduct multiple dose inoculums to determine a dose response relationship and whether dose plays a role in the observed differences in pathogenicity. Finally, our study characterizes the pathogenicity of a single LA-*S. aureus* nasal carriage isolate, whereas isolates within the LA-*S. aureus* CC398 and CC9 lineages display considerable diversity. Thanh-Thao et al. reports a conservation of core-genome elements, such as *spa type,* between nasal carriage isolates within individual, but more heterogeneity between isolates for mobile genetic element (MGE) encoded genes, such as *scn* and AMR genes.[100] Differences in pathogenicity between closely related isolates of *S. aureus* is well established, and future studies should compare multiple isolates of each lineage to make confident lineage related conclusions.

CONCLUSIONS

The results of this study provide new insights into the pathogenicity of emerging LA-*S. aureus* strains that are commonly contracted by IHO workers and may have

potential for dissemination into the community.[10] The increased disease and bacterial burden observed in the mice in response to the LA-*S. aureus* skin infection compared to a highly virulent CA-MRSA skin infection indicate an importance for occupational and public health. The WGSA suggests that a subset of VF genes involved in the pathogenesis of *S. aureus* SSTIs was conserved across CA-MRSA and LA-*S. aureus* isolates, while LA-*S. aureus* isolates carried a greater frequency and diversity of AMR genes encoding for resistance to antibiotics that are critically important for human health.[72] Therefore, future research efforts to study, monitor and determine virulence mechanisms, host immune responses and response to treatment in humans that suffer SSTI caused by LA-*S. aureus* strains should be considered.

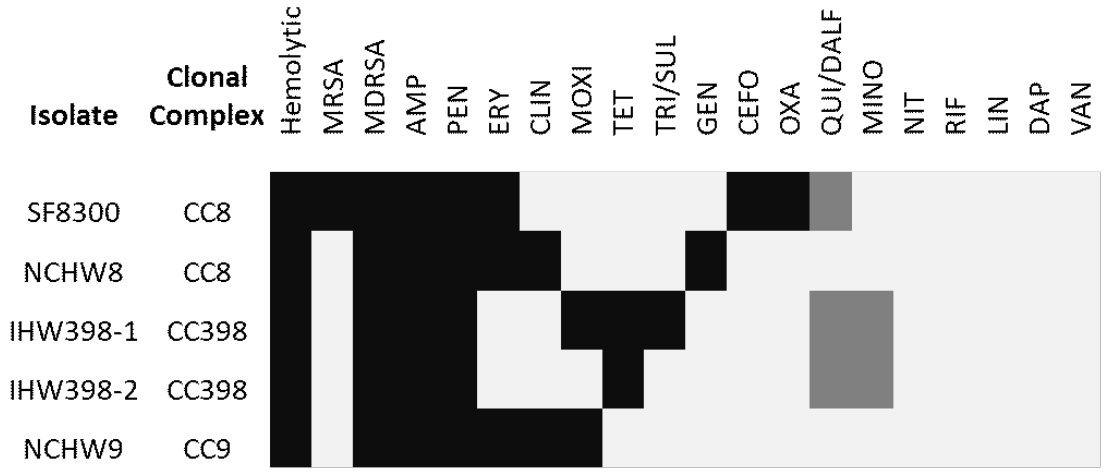


Figure 2.1. Hemolytic activity and antibiotic susceptibilities of LA-*S. aureus* and CA-MRSA. Hemolysis and antibiotic susceptibility testing results are shown for each *S. aureus* isolate Hemolytic activity was measured as a binary variable, and defined as the formation of a zone of hemolysis after 24 hours of growth on blood sheep agar. MRSA was defined as cefoxitin or oxacillin resistant or positive for the *mecA* or *mecC* gene. MDRSA was defined as resistant or intermediate to at least three classes of antibiotics. For the hemolytic, MRSA, and MDRSA categories, black = positive and white = negative. For antibiotic susceptibility categories, black = resistant, grey = intermediate, and white = susceptible.

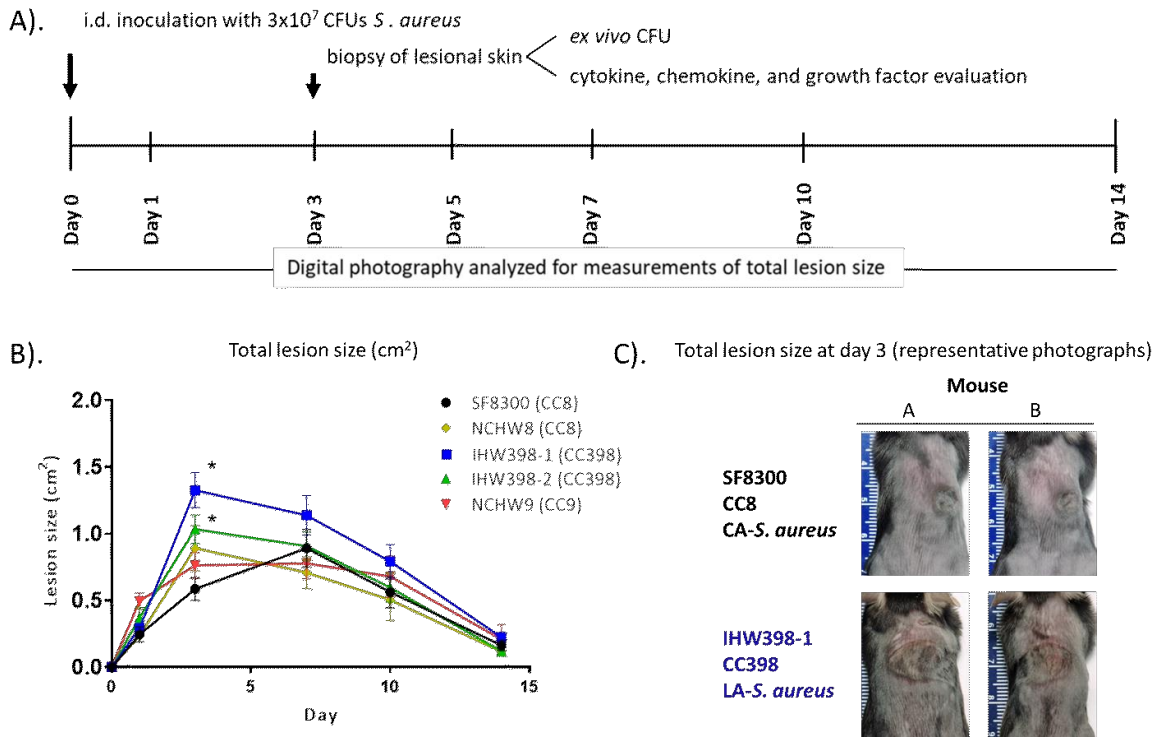


Figure 2.2. LA-*S.* aureus-infected mice develop larger lesion sizes compared to CA-MRSA. (A) Schematic of mouse model of SSTI. (B) Mean total lesion size (cm^2) ± SEM. (C). Representative photographs of the lesions at day 3 for two mice. 1*, $p < 0.05$ = each LA-*S. aureus* strain (n=10/group) versus CA-MRSA SF8300 (n=20), as calculated by a two-tailed Student's *t*-test. *Note.* LA = livestock-associated. CA = community-associated. MRSA = methicillin-resistant *S. aureus.* i.d. = intradermal. CFU = colony forming unit.

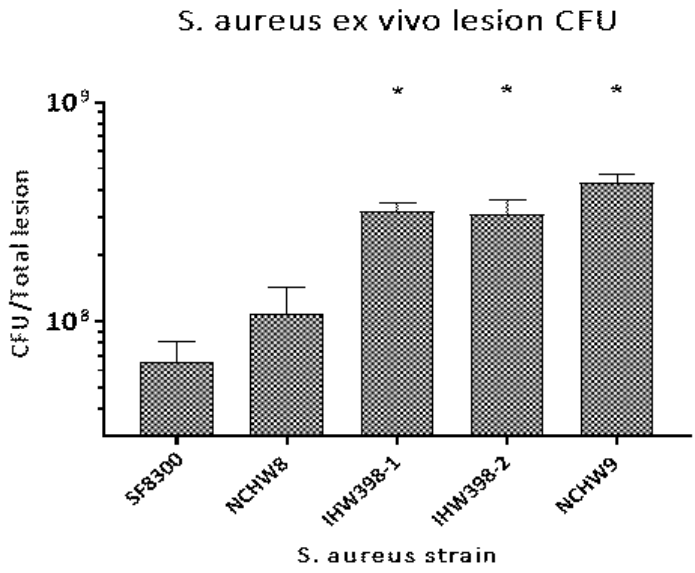
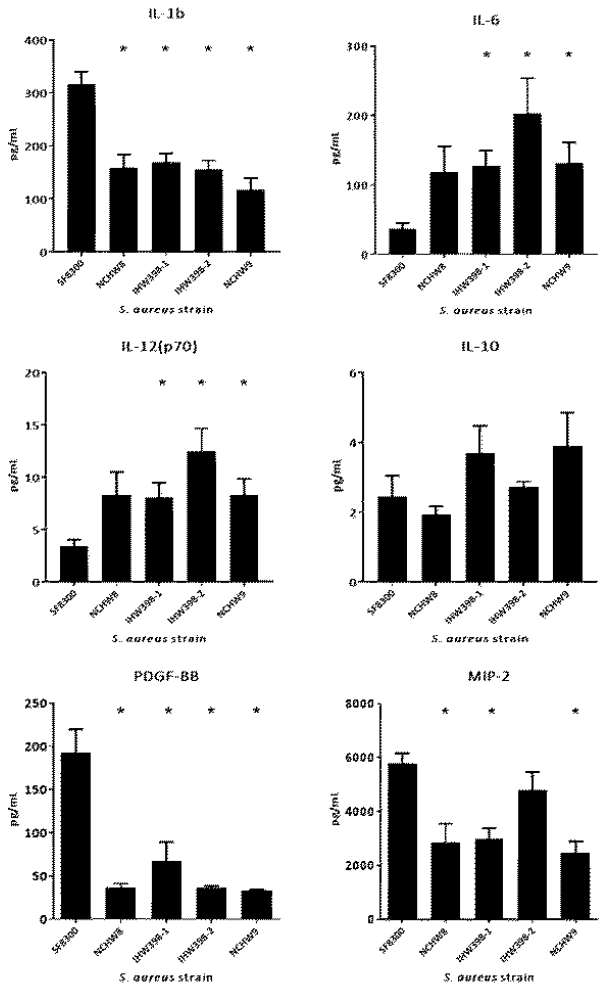


Figure 2.3. ***Ex vivo*** **colony forming units (CFU) in LA-*S. aureus* and CA-MRSA infected skin.** On day 3, homogenates of infected skin were assayed for *ex vivo* CFU levels. *Ex vivo* CFU ± SEM. *, $p < 0.05$, between LA-*S. aureus* strains versus SF8300 (n=5/group), as calculated by a two-tailed Student's *t*-test.

A)



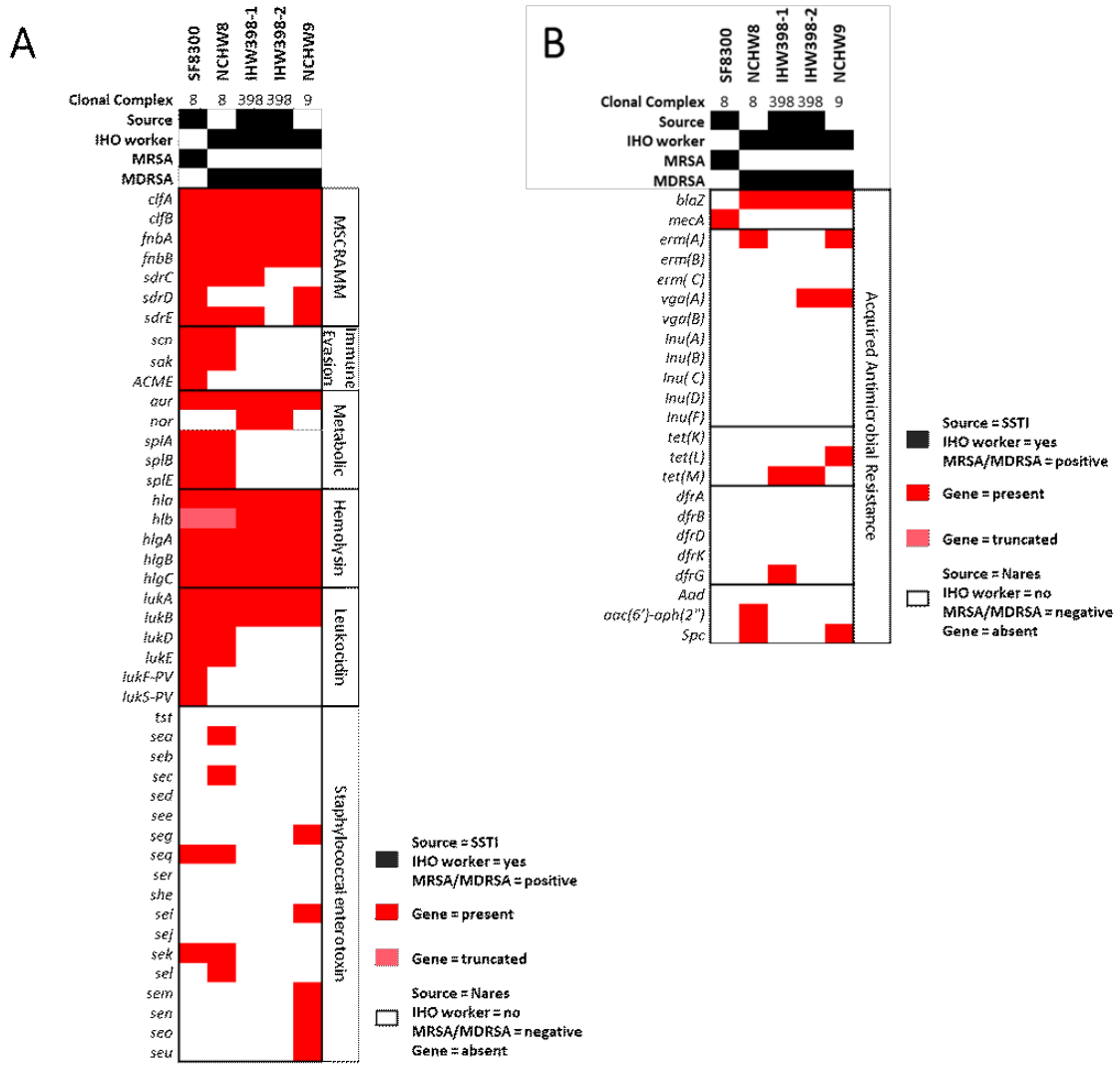
B)

	NCHW8	IHW398-1	IHW398-2	NCHW9
IL-1B	*	*	**	**
IL-6		*	*	*
LIF				
IL-10				
IL-12(p40)		**		
IL-12(p70)		**	**	**
IL-13	**		*	
IL-15		*		*
IL-18				**
TNF-a				
M-CSF				
MIG				
MIP-2	*	**		**
PDGF-BB	*	*	*	*
FGF-basic				*
VEGF				
IL-4	N/A	N/A	N/A	N/A
IL-5	N/A	N/A	N/A	N/A
IL-17A	N/A	N/A	N/A	N/A
IFN-g	N/A	N/A	N/A	N/A
MPO				

* = significantly greater than SF8300 infected mice

* = significantly lower than SF8300 infected mice

<u>Figure 2.4.</u> Cytokine, chemokine and growth factor protein levels in LA-*S. aureus* and CA-MRSA infected skin. On day 3, homogenates of infected skin were assayed for *ex vivo* protein levels of host response proteins. (A) Mean protein levels (pg/mL homogenate) ± SEM. *, $p < 0.04$, between LA-*S. aureus* strains versus SF8300, as calculated by a two-tailed Student's *t*-test. (B) Summary of LA-*S. aureus* protein levels compared to CA-MRSA (SF8300) protein levels for all 21 proteins. *, $p < 0.04$; **, $p < 0.002$ = statistically significant difference via two-tailed students *t*-test compared to reference SF8300 strain. Typically, 5 mice per *S. aureus* strain were used.



<u>Figure 2.5.</u> Virulence factor and AMR genes in LA-*S. aureus* and CA-MRSA. The presence of a genome hit (indicated by red shading) was defined >85% gene identification similarity and >60% gene length similarity and confirmed by manual inspection. (A) Microbial surface components recognizing adhesive matrix molecules (MSCRAMM), human immune evasion, metabolic enzyme, hemolysin, leucocidin/toxin, and *Staphylococcal* enterotoxin gene genome hits generated via VirulenceFinder 1.5 or ABRicate tool. (B) Acquired antimicrobial resistance gene genome hits generated via ResFinder 3.0. *Note.* LA = livestock-associated. CA = community-associated. MRSA = methicillin-resistant *S. aureus.* MDRSA = multidrug-resistant *S. aureus.*

CHAPTER 3

Development and application of antibody-based biomarkers of *Staphylococcus aureus* exposure, colonization, and infection in oral fluid among industrial hog operation workers and their household contacts in North Carolina, United States

ABSTRACT

Introduction. Recent advances in our understanding of the adaptive immune response to *S. aureus* have lead to the investigation of antibody-based biomarkers associated with *S. aureus* colonization and infection. While epidemiological studies provide strong support for occupational exposure to *S. aureus* among industrial hog operation (IHO) workers, to our knowledge, no studies have aimed to measure IgA and IgG antibodies against candidate *S. aureus* antigens in a population of IHO workers and their household contacts. Furthermore, no studies, to our knowledge, have evaluated *S. aureus* antigen-specific IgA and IgG antibody levels in oral fluids.

Objectives: The objectives of this study were to measure IgA and IgG antibody levels against staphylococcal complement inhibitor (SCIN), clumping factor A (ClfA), and alpha toxin (AT) in OF collected from IHO workers and their household contacts and examine these biomarkers' association with *S. aureus* nasal carriage outcomes,self-reported skin and soft tissue infection (SSTI), and personal protective equipment (PPE) use.

Methods. An OF multiplex *S. aureus* Luminex enzyme immunoassay (EIA) was developed. Magnetic microsphere bead coupling was confirmed for SCIN, ClfA, and AT antigens. The multiplex Luminex EIA for IgA and IgG specific to SCIN, ClfA, and AT antigens was applied to OF samples collected from a cohort of IHO workers and their household contacts in North Carolina (NC), United States (USA). Associations were assessed between median fluorescence intensity (MFI) for IgA and IgG in OF specific to

SCIN, ClfA, and AT and *S. aureus* nasal carriage outcomes, and self-reported SSTI and facemask use.

Results. OF IgA and IgG antibody levels against SCIN, ClfA, and AT among children (7-17 years) were significantly lower than those among adults. OF IgA antibody levels remained elevated among elderly adult age groups (≥50 years of age), whereas OF IgG antibody levels waned at elderly ages. Anti-ClfA IgA and IgG antibody levels were significantly elevated among IHO workers who carried *S. aureus*, multidrug-resistant *S. aureus* (MDRSA), and tetracycline-resistant *S. aureus* (tet[R]-*S. aureus*) intranasally. These associations were attenuated among IHO workers who reported they always or sometimes wore a facemask. Anti-ClfA IgA levels were positively and anti-SCIN levels were negatively associated with self-reported SSTI.

Conclusions. An OF-based *S. aureus* multiplex immunoassay was developed that can be employed in occupational and community settings to measure early biologic antibody-based response to *S. aureus* exposure. IHO workers who are occupationally exposed to IHO-derived *S. aureus* subpopulations, including MDRSA and tet[R]-*S. aureus*, displayed elevated antigen-specific IgA and IgG antibodies against *S. aureus* in OF. OF antibodies against ClfA may serve as a promising biomarker of occupational exposure and progression to early biologic effects related to *S. aureus* acquisition among IHO workers.

INTRODUCTION

Recent advances in our understanding of the adaptive immune response to *S. aureus* have lead to the investigation of antibody-based biomarkers associated with *S. aureus* colonization and infection.[27,99] While a number of antigen-specific antibodies against *S. aureus* antigens may serve as candidate biomarkers of *S. aureus* exposure,[99,101] multiple groups have shown elevated serum IgA and IgG antibody levels against clumping factor A (ClfA) and alpha toxin (AT) in response to *S. aureus* colonization.[27,99,101] Furthermore, anti-AT IgG antibody levels are consistently elevated among clinical populations experiencing *S. aureus* SSTI,[27] bacteremia,[102] and exposure through injection drug use.[42] A robust serum IgA and IgG signal against Staphylococcal Complement Inhibitor (SCIN) has also been demonstrated among populations nasally colonized with *S. aureus*[99] and experiencing *S. aureus* bacteremia,[101] indicating that SCIN is a highly immunogenic *S. aureus* protein. Thus, IgA and IgG antibodies directed against *S. aureus* antigens that play a role in adhesion, immune evasion, and pathogenicity, may serve as antibody-based biomarkers of early biological effect in response to *S. aureus* exposure.

A growing body of evidence suggests that having frequent contact with, or living near, swine raised on industrial hog operations (IHO) is associated with increased *S. aureus* exposure, including carriage of methicillin- (MRSA) and multidrug- (MDRSA) resistant *S. aureus*.[10,11,14–16,103] Furthermore, individuals exposed to livestock-associated (LA-) *S. aureus* are at risk of developing mild-to-severe infections, including skin and soft tissue infections (SSTIs) and blood stream infections.[16,25] In particular, IHO-workers in North Carolina (NC), United States (USA) who are occupationally exposed to swine

are at an increased risk of carrying *S. aureus* intranasally,[10,14,15] including MDRSA,[14,15] tetracycline resistant *S. aureus* (tet[R]-*S. aureus*) ,[14,15] and LA-*S. aureus*.[14,15] While epidemiologic studies provide strong support for an occupational exposure to *S. aureus* in the IHO environment, to our knowledge, no studies have aimed to measure IgA and IgG antibodies against candidate *S. aureus* antigens in a population of IHO-workers and their household contacts in the USA

While most studies aimed to understand the anti-*S. aureus* humoral response are focused on serum, oral fluids (OF) represent a noninvasive biological fluid with scientific and clinical potential in the context *S. aureus* colonization and infection. The potential for utilizing OF in longitudinal epidemiologic studies, particularly those focused on understanding the temporal kinetics of transient exposure-response dynamics, has prompted the development and application of OF-based immunoassays to measure the burden of human exposure to environmental and clinical pathogens.[104,105] The two dominant antibody classes that operate in OF are secretory IgA (SIgA), produced by local plasma cells in OF glands, and IgG, derived primarily from passive leakage of blood via the gingival crevicular epithelium.[106] A growing body of evidence now supports that OF IgA and IgG can dependably reflect mucosal and systemic immune activity,[107] and it has been previously reported that anti-*S. aureus* antibody levels (IgA and IgG) in secretions from the nasal mucosa correlate with levels in serum.[102] To our knowledge, IgA and IgG antibodies against *S. aureus* antigens in OFs remains entirely unexplored. Considering the consistent observation of elevated serum anti-ClfA, anti-AT, and anti-SCIN antibody levels among *S. aureus* exposed populations,[27,42,101,102] we parsimoniously chose to probe

OF samples collected from IHO workers and their household contacts in NC, USA for IgA and IgG antibodies against ClfA, AT, and SCIN.

The objectives of this study were to: 1) Develop an OF multiplex *S. aureus* EIA, 2) study the relationship between age and OF anti-ClfA, anti-AT, and anti-SCIN IgA and IgG levels among IHO workers and their household contacts, and determine if this relationship is consistent with previously published research, 3) determine the relationship between *S. aureus* nasal carriage outcomes (MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus*) and OF IgA and IgG levels against SCIN, ClfA, and AT among IHO-workers and their household contacts, 4) understand how the relationship between OF IgA and IgG levels against ClfA, AT, and SCIN are modified by protective facemask use[14] among IHO-workers; and 5) evaluate associations between OF IgA and IgG levels against SCIN, ClfA, and AT and self-reported SSTI among IHO workers and their household contacts.

METHODS

Population and sample collection.

For this study, data and samples were collected from IHO-workers and their adult and minor household contacts who participated in a prospective cohort study in North Carolina (NC), USA between October 2013 and February 2014.[14,15] IHO-workers were recruited who fit the following inclusion criteria: resided in NC, currently worked at an IHO, could speak English or Spanish, and was at least 18 years of age. Participants were considered IHO-workers only if they were employed in the production of live pigs. Individuals employed in other types of food animal production, such as meat processing,

were not included in the study. Up to three (3) IHO-worker household contacts were allowed to participate, and they were eligible to participate if they were at least seven years of age and spoke English or Spanish.

At enrollment, participants completed a baseline questionnaire. Participants were followed for 4 months after baseline, and completed up to eight questionnaires every 2 weeks (biweekly) throughout the study. The baseline questionnaire captured demographic information, household-level characteristics, and time-invariant activities that could be related to *S. aureus* exposure. At baseline and at each biweekly visit, participants provided a self-collected BD BBL CultureSwab (BD Diagnostics) from both of their nares and an oral fluid sample (Oracol S10 saliva swab, Malvern Medical Development, Worcester, UK). To collect OF samples, participants were first instructed to rinse their mouth with cold water and then brush the Oracol along their gum line for one minute. Following collection, OF samples were stored at 4^{o}C until reaching the lab. Once at the lab, the Orocol sponge of each OF collection devise was turned upside down (sponge down) using sterile forceps, and was centrifuged for 10 mins at 1,500 g to spin the OF out of the sponge. The sponge was then removed, OF was transferred into cryovials, and were then stored at -80^{o}C.

***Detection of* S. aureus.**

Each self-collected BD BBL CultureSwab was clipped into 1mL of phosphate-buffered saline (PBS) and vortexed for 60 seconds. 100ul of this eluate was plated onto CHROMagar™ Staph aureus (BD, Franklin Lakes, ND) and incubated at 37^{o}C for 24 hours. After 24 hours of incubation, at least two colonies with morphological characteristics indicative of *S. aureus* were re-streaked to isolation on a new CSA plate. If

direct plating did not result in any colony formation with *S. aureus* morphology, swabs and the remaining PBS were enriched in 10mL of Mueller-Hinton broth with 6.5% NaCl overnight. 10ul of the enriched broth was streaked onto both CSA and Baird Parker plates to increase sensitivity of detection.[47] Up to two colonies with morphology indicative of *S. aureus* were re-streaked to isolation on a new CSA plate. Presumptive colonies were archived in brain heart infusion broth with 15% (w/v) glycerol at -80C.

Assessment of antibiotic susceptibility.

Antibiotic susceptibility testing (AST) was completed by the Clinical Microbiology Laboratory at the Johns Hopkins Hospital. One isolate from each *S. aureus* positive nasal swab was assessed for susceptibility to a panel of antibiotics (Supplementary information: Table 3S.1) using the Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD).

Molecular characterization of* S. aureus *isolates.

A crude DNA extraction was performed on each isolate using a previously described protocol.[14] *S. aureus spa, scn, mecA, and mecC* genes were amplified using multiplex PCR as previously described.[14] PCR products were visualized on 2% agarose gels stained with ethidium bromide. Colonies positive for the *spa* gene were classified as *S. aureus*. All *S. aureus* confirmed isolates were characterized by *spa* typing using the Ridom StaphType software and the Ridom SpaServer (http://spa.ridom.de/index.shtml).[108] We did not perform multi-locus sequence typing (MLST). Putative MLST for each isolate was assigned based on previously reported *spa* types associated with *S. aureus* CC398 and CC9.[14]

Definition of* S. aureus *subpopulations.

In the current study we consider a *S. aureus* positive nasal swab as a *S. aureus* positive nasal carriage event. If no *S. aureus* could be isolated by direct plating or after enrichment, we consider this a *S. aureus* negative carriage event. Because previous studies have reported an increased risk of MDRSA, tet[R]-*S. aureus,* and LA-*S. aureus* nasal carriage among IHO-workers in NC, USA,[9,14,15] in the current study we consider MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus* as IHO-derived *S. aureus* subpopulations. MDRSA was defined as *S. aureus* isolates resistant to three or more classes of antibiotics, based on clinical MIC threshold values (Supplementary information: Table 3S.1). Tet[R]-*S. aureus* was defined as *S. aureus* isolates resistant to tetracycline, based on clinical MIC threshold values (Supplementary information: Table 3S.1). There is currently no established marker for LA-*S. aureus*. Strain type CC398, strain type CC9, an absence of the *scn* gene, and phenotypic resistant to tetracycline have been used previously as markers of LA-*S. aureus*.[14] In the current study, we consider isolates that are strain type CC398, strain type CC9, *scn(-)*, or phenotypically resistant to tetracycline as LA-*S. aureus*.

Multiplex immunoassay for* S. aureus *antigen-specific IgA and IgG in oral fluids (OF).

Recombinant *S. aureus* proteins for Staphylococcal Complement Inhibitor (SCIN), and Clumping Factor A (ClfA) were developed and purchased from MyBioSource (MyBioSource Inc., San Diego, CA). Both recombinant SCIN and ClfA contained an N-terminal 6xHis tag. Natural *S. aureus* Alpha-toxin protein (AT) protein was sourced from LifeSpan Biosciences (LifeSpan BioSciences Inc., Seattle, WA).

Each protein was covalently coupled to unique magnetic microparticle sets, hereafter referred to as "bead sets" (MagPlex microspheres, Luminex) as described previously. [109]Successful coupling of each bead set was confirmed using a serial dilution of monoclonal mouse antibodies against each *S. aureus* protein (SCIN, ClfA, AT), followed by R-phycoerythrin (PE)-labelled anti-mouse antibody. Coupling of each bead set was also confirmed using convalescent sera from mice intradermally inoculated with *S. aureus*. Coupling of *S. aureus* proteins to bead sets was considered confirmed if bead sets revealed a fluorescent signal of >18,000 mean fluorescence intensity (MFI).

We selected baseline (visit 0), biweekly visit 1 (visit 1), and biweekly visit 8 (visit 8) oral fluid (OF) samples from all participants to probe with ClfA, AT, and SCIN coupled bead sets. OF samples were centrifuged for 5 minutes at 10,000g and 20ºC, resulting in a cell pellet and varying volumes of OF samples returning less than 100ul of volume after centrifugation were excluded from the sample set. 10ul of the oral fluid supernatant was added to 40ul of assay buffer (PBS with 0.05% Tween20 and 1% bovine serum albumin) containing 1500 beads of each bead set (SCIN, ClfA, AT) per microplate well. The plate was covered and incubated at room temperature for 1 hour on a plate shaker at 500 rpm. Beads were washed 3 times and then incubated with 50ul of PE-labelled anti-human IgA diluted 1:100 in assay buffer for 1 hour on a plate shaker at 500 rpm. Beads were washed again and then suspended in 100ul assay buffer. The fluorescence signal was measured on a Bio-Plex 200 instrument (Bio-Rad). The same process was repeated, but using PE-labelled anti-human IgG diluted 1:100 for the secondary incubation. Two (2) blanks were included on each plate to subtract

background, and ~10% of OF samples were tested in duplicate to determine intra-assay variability.

Statistical analysis.

We first characterized the association between age and antigen-specific IgA and IgG antibody levels in OF samples of participants. A total of 42 observations were missing data on participant age (8.5%) and were excluded from all subsequent analysis. The age variable was grouped into five categories as follows: 1). Children 7-17 years of age; 2). Adults 18-29 years; 3). Adults 30-39 years; 4). Adults 40-49 years; and 5). Elderly 50-82 years. MFI values for OF IgA and IgG antibody levels were $\log_{10}$ transformed prior to analysis. To examine the relationship between age and change in antigen-specific OF IgA and IgG levels, we compared each adult age category to the reference group of children 7-17 years of age. Beta coefficients and 95% confidence intervals were estimated using generalized linear models, clustering at the individual level to account for a lack of independence of repeated measures. Two-way scatter plots and LOWESS curves were used to examine potential non-linearity of the relationship between continuous age and antigen-specific IgA and IgG levels in OF (Figure 3S.1). Because prior studies demonstrated an age-dependent variability in antigen-specific IgA and IgG in serum, all regression models were adjusted for continuous age.

Similar to prior studies of *S. aureus*-specific IgA and IgG[27], children (7-17 years of age) were excluded from regression models aimed at evaluating the association between *S. aureus* nasal carriage outcomes (*S. aureus*, MDRSA, tet[R]-*S. aureus*, or LA-*S. aureus*) as the independent variable and continuous $\log_{10}$ transformed OF IgA and IgG

MFI values (of SCIN, ClfA, AT) as the dependent variable. Log_{10} transformed average OF IgA and IgG MFI values were compared between *S. aureus* carriage positive events and non-carriage events. Age-adjusted beta coefficients and 95% confidence intervals (CIs) of OF IgA or IgG MFI were estimated using generalized linear models, clustering to account for a lack of independence of repeated measures within participant, comparing *S. aureus* positive carriage events to non-carriage events. We analyzed pooled effect estimates among IHO-workers and household member adults combined and also stratified effect estimates among IHO-workers and adults separately.

To examine variability in the association between *S. aureus* carriage outcomes (*S. aureus*, MDRSA, tet[R]-*S. aureus*, or LA-*S. aureus*) and log_{10}-transformed OF IgA and IgG MFI values by facemask use we evaluated regression models among IHO worker only stratified by self-reported frequency of facemask use at baseline defined as: always, sometimes, never. Binary *S. aureus* nasal carriage outcomes were modeled as the independent variable and log_{10} transformed OF IgA and IgG MFI values for each *S. aureus* antigen target (SCIN, ClfA, AT) were included as continuous dependent variables, within each stratum of facemask use.

To evaluate the robustness of the relationship between IHO-derived *S. aureus* (MDRSA, tet[R]-*S. aureus*, or LA-*S. aureus*) exposure and the magnitude of OF IgA and IgG antibody levels, we conducted three sensitivity analyses. In regression models of the association between *S. aureus* nasal carriage and log_{10} transformed OF IgA and IgG MFI values for each *S. aureus* antigen target (SCIN, ClfA, AT) we first excluded all MDRSA nasal carriage events, second, excluded all tet[R]-*S. aureus* carriage events, and finally, we excluded all LA-*S. aureus* nasal carriage events. These sensitivity analyses were

designed to determine whether excluding specific *S. aureus* subpopulations (some of which are putatively livestock-associated) would lead to an attenuation of effect estimates.

To determine whether OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels might serve as improved biomarkers of SSTI risk relative to *S. aureus* nasal carriage outcomes, we estimated prevalence ratios (PRs) and 95% confidence intervals (CIs) of self-reported SSTI via log-binomial regression models that included covariates for $\log_{10}$ transformed anti-SCIN, anti-ClfA, and anti-AT antibody levels (IgA and IgG each evaluated separately) as well as *S. aureus* nasal carriage outcomes (included one at a time along with all three anti-*S. aureus* antibody levels). Because 50% (6/12) of self-reported SSTIs were observed in children, all participants were included in this analysis. Log-binomial regression models were estimated, clustering at the individual level to account for a lack of independence of repeated measures within participant. We analyzed pooled effect estimates among IHO-workers, adults, and children. All statistical analyses were completed using Stata version 14.2 (StataCorp, LLC, College Station, Texas).

RESULTS

Sample selection.

We probed a total of 496 OF samples, collected at 3 different time points (baseline visit 0, biweekly visit 1, and biweekly visit 8), from 179 participants for anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels. Ninety five IHO-workers contributed 255 OF samples, 27 adults contributed 67 OF samples, and 52 minors

contributed 149 OF samples. A total of 25 observations (5%) were missing host data, and a total of 42 observations (8.5%) were missing age data. Out of the 179 participants, 83% (n=148) contributed an OF sample at all three time points, 13% (n=22) contributed an OF sample at two time points, and 4% (n=8) contributed a single OF sample. Anti-SCIN IgA and IgG fluorescent signals were undetectable in 3 OF samples each. Anti-ClfA IgA and IgG fluorescent signal were undetectable in 3 and 4 OF samples respectively. Anti-AT IgA and IgG fluorescent signal were undetectable in 3 and 4 OF samples, respectively.

Relation of age with OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels.

We probed 451 saliva samples from 166 participants ranging in ages between 7 and 82 for anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels. Overall, OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG signals increased until about age 40 followed by a decrease at older ages (Figure 3S.1), which is consistent with previous studies that measured antibodies in serum.[27]

Because previous literature had reported significantly reduced serum anti-AT IgG antibody levels among infants and children,[27] we compared the OF IgA and IgG signal in children to those of IHO workers and adults. All beta coefficient, confidence interval, and *p* values for age relationships are provided in Table 3S.2. OF anti-SCIN IgA and IgG antibody levels significantly trended towards elevated among IHO-workers and adults ages 19-49 compared with children 7-17 years of age (Figure 3.1A, Table 3S.2). While anti-SCIN IgA levels were significantly elevated in the oldest age group of IHO workers and adults compared to children, this was not the case for anti-SCIN IgG levels (Figure 3.1A, Table 3S.2). OF anti-ClfA IgA levels were significantly elevated among IHO

workers and adults 30 years of age or older compared to children 7-17 years of age (Figure 3.1B, Table 3S.2). OF anti-ClfA IgG levels were significantly elevated among IHO-workers and adults between the ages of 19-39 compared to children 7-17 years of age (Figure 3.1B, Table 3S.2). OF anti-ClfA IgG levels among IHO workers and adults of 40 years of age or older were not significantly elevated compared with children 7-17 years of age (Figure 3.1B, Table 3S.2). OF anti-AT IgA and IgG antibody levels were significantly elevated among IHO-workers and adults ages 19-49 compared with those of children 7-17 years of age (Figure 3.1C, Table 3S.2). While OF anti-AT IgA levels were significantly elevated in the oldest age group of IHO-workers and adults compared to children, this was not the case for anti-AT IgG levels (Figure 3.1C, Table 3S.2).

S. aureus ***nasal carriage and OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels.***

Previous studies have reported a modest population level increase in serum anti-ClfA IgA antibody levels[99], and serum anti-AT IgG antibody levels among *S. aureus* colonized adults compared to non-colonized adults.[27] To determine if *S. aureus* nasal carriage is associated with increased OF IgA and IgG antibody levels, we compared OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG levels between *S. aureus* carriage (n=178) and non-carriage (n=142) events. Because previous studies limited analysis to adult aged participants,[27,99] children were excluded from this and all subsequent analyses. When IHO-workers and adults were grouped together (n=320), we detected a trend for elevated OF anti-ClfA IgG (beta coefficient=0.131, 95% CI: -0.0004, 0.262) and anti-AT IgG (beta-coefficient=0.105, 95% CI: -0.007, 0.217) antibody levels when IHO-workers and adults were positive for nasal carriage with *S. aureus,* although these differences did

not reach statistical significance (Table 3.2). OF anti-SCIN IgG and all OF IgA antibody levels did not appear to show any association with *S. aureus* nasal colonization (Table 3.2). We then analyzed IHO-workers and adults as separate groups, to account for different frequency and magnitude of occupational exposure to *S. aureus* and the IHO environment. Among IHO-workers only (n=250), we detected a significant trend for elevated OF anti-ClfA IgG antibody levels when IHO-workers were positive for *S. aureus* nasal carriage (beta coefficient=0.156, $p < 0.031$) (Table 3.2). We also detected a trend for elevated OF anti-ClfA IgA antibody levels when IHO-workers were positive for *S. aureus* nasal carriage (Table 3.2), albeit not reaching statistical significance (beta coefficient = 0.131, 95% CI: -0.016, 0.279). We did not detect any association between OF anti-SCIN and anti-AT IgG or IgA antibody levels and *S. aureus* nasal carriage among IHO-workers (Table 3.2). Among adults only, we did not detect any association between any of the OF IgA and IgG signals and *S. aureus* nasal carriage (Table 3.2). Taken together, nasal carriage of *S. aureus* is associated with a trend toward elevated OF anti-ClfA IgA and IgG antibody levels among IHO-workers but not among adults who are household contacts of IHO-workers, consistent with the elevated degree of acute *S. aureus* exposure experienced by IHO-workers.[15,17]

Nasal carriage of IHO-derived* S. aureus *subpopulations (LA*-S. aureus, *tet[R]*-S. aureus, *and MDRSA) and OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels.

In our previous work we showed that IHO workers were specifically at an increased risk of carrying MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus* compared to adults without any exposure to livestock, suggesting an occupational exposure to IHO-

derived *S. aureus*.[9,10,15] We thus sought to determine if exposure to IHO-derived subpopulations of *S. aureus,* defined as MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus*, were associated with elevated OF IgA and IgG antibody levels. To determine if nasal carriage of IHO-derived *S. aureus* subpopulations was associated with increased OF IgA and IgG antibody levels, we compared OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG levels between IHO-derived *S. aureus* carriage and non-carriage events.

When IHO-workers and adults were analyzed together (n=308), IHO-workers and adults who carried MDRSA intranasally displayed a significant trend towards elevated OF anti-SCIN and anti-ClfA IgA antibody levels (anti-SCIN IgA: beta coefficient = 0.18, 95% CI: 0.042, 0.318; anti-ClfA IgA: beta coefficient = 0.281, 95% CI: 0.099, 0.463) compared to when IHO-workers and adults did not carry any *S. aureus* (Table 3.2). We detected a trend towards elevated OF anti-SCIN, anti-ClfA, and anti-AT IgG when IHO-workers and adults carried MDRSA intranasally, but this did not reach statistical significance (Table 3.2). When IHO-workers and adults carried tet[R]-*S. aureus*, we detected a significant trend towards elevated OF anti-ClfA IgG antibodies compared to when IHO workers and adults did not carry any *S. aureus* (beta coefficient=.198, 95% CI: .020, .376). OF anti-ClfA IgA trended towards elevated levels among tet[R]-*S. aureus* carriage events, but did not reach statistical significance (beta coefficient=.192, 95% CI: -0.011, 0.396). We did not detect any antibody trend associated with carriage of LA-*S. aureus* compared to non-carriage when IHO-workers and adults were analyzed together (Table 3.2).

When analyzed among IHO-workers only (n=250), we detected a significant trend towards elevated OF anti-ClfA IgA (beta coefficient=.278,95% CI: 0.077, .479) and anti-

ClfA IgG (beta coefficient=.191,95% CI: 0.003, 0.378) antibody levels when IHO-workers carried MDRSA intranasally compared to when IHO-workers did not carry any *S. aureus* (Table 3.2). Although OF anti-SCIN IgA antibody signal displayed a trend toward elevated levels when IHO-workers carried MDRSA intranasally, this association was not statistically significant (beta coefficient=.143,95% CI: -0.017, 0.302). We also detected a trend towards elevated OF anti-ClfA IgA (beta coefficient=.203, 95% CI: -0.016, .424) and IgG antibodies (beta coefficient=.185, 95% CI: -0.016, .0.386) when IHO-workers carried tet[R]-*S. aureus* compared to when they did not carry any *S. aureus*, which also did not reach statistical significance (Table 3.2).

When analyzed among adults only (n=59), we detected a significant trend towards elevated OF anti-SCIN IgA antibody levels when adults carried MDRSA intranasally compared to when adults did not carry any *S. aureus* (beta coefficient=.349,95% CI: 0.157, 0.541) (Table 3.2). We also detected a significant trend towards elevated OF anti-SCIN IgA (beta coefficient=.269,95% CI: 0.028, 0.510)) and IgG (beta coefficient=.291,95% CI: 0.026, 0.556) antibody levels when adults carried tet[R]-*S. aureus* compared to when adults did not carry any *S. aureus.*

Modification of the association between* S. aureus*, LA*-S. aureus*, tet[R]*-S. aureus*, and MDRSA colonization and OF anti-SCIN, anti-ClfA, and anti-AT antibody levels by self-reported facemask use among IHO-workers.

In our previous work, we show that consistent facemask use among IHO-workers is associated with decreased exposure to MDRSA and tet[R]-*S. aureus*.[14] To determine how facemask use modified the association between IHO-derived *S. aureus* carriage

(MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus*) and OF IgA and IgG antibody levels, we compared OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG levels between positive and negative IHO-derived *S. aureus* subpopulation carriage events, stratified by self-reported facemask use at baseline (never, sometimes, or always). Among IHO-workers who reported sometimes (n=108) or always (n=83) wearing a facemask, we did not detect any trend in OF IgA or IgG antibody levels associated with carriage of any type of IHO-derived *S. aureus* subpopulation (Table 3.3). IHO-workers who reported never wearing a facemask (n=53) displayed a significant trend towards elevated OF anti-SCIN IgA and IgG antibody levels when carrying MDRSA (anti-SCIN IgA: beta coefficient=.221, 95% CI: 0.014, 0.428); anti-SCIN IgG: beta-coefficient=.376,95% CI: 0.098, 0.653)) or tet[R]-*S. aureus* (anti-SCIN IgA: beta coefficient=.176,95% CI: 0.005, 0.347; anti-SCIN IgG: beta coefficient=.368,95% CI: 0.070, 0.665) compared to when not carrying any *S. aureus* (Table 3.3). IHO-workers who reported never wearing a facemask also displayed a significant trend towards elevated anti-ClfA IgG antibody levels when carrying any *S. aureus* (anti-ClfA IgG: beta coefficient=.281,95% CI: 0.068, 0.493), and elevated anti-ClfA IgA and IgG antibodies when carrying MDRSA (anti-ClfA IgA: beta coefficient=.473,95% CI: 0.218, 0.728); anti-ClfA IgG: beta coefficient=.437, 95% CI: 0.090, 0.785), tet[R]-*S. aureus* (anti-ClfA IgA: beta coefficient=.418, 95% CI: 0.156, 0.681); anti-ClfA IgG: beta coefficient=.513,95% CI: 0.180, 0.845), or LA-*S. aureus* (anti-ClfA IgA: beta coefficient=.356, 95% CI: 0.114, 0.599; anti-ClfA IgG: beta coefficient=.317,95% CI: 0.046, 0.589) compared to when not carrying any *S. aureus*. We also detected a significant trend towards elevated OF anti-AT IgG antibody levels when IHO-workers carried tet[R]-*S. aureus* compared to when they did not, among IHO-

workers who reported never using a facemask (beta coefficient=.290,95% CI: 0.045, 0.536) (Table 3.3).

Taken together, facemask use by IHO-workers modified the effect of exposure to IHO-derived *S. aureus* subpopulations on OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels. This is noted by a robust association of elevated OF IgA and IgG antibody levels among IHO-workers who reported never using a facemask and carried IHO-derived *S. aureus* compared to when they did not carry *S. aureus*. This is also supported by attenuated associations among IHO-workers who reported sometimes or always using a facemask.

OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels and self-reported skin and soft tissue infection (SSTI).

In regression models that included both antibody-based and *S. aureus* nasal carriage outcome biomarkers we observed an attenuation of the association between each of the *S. aureus* nasal carriage outcomes and self-reported SSTI. For example, the *S. aureus* nasal carriage outcome SSTI PR was 1.75 (95% CI: 0.69, 4.45) (Table 3.4). For the MDRSA, tet[R]-*S. aureus,* and LA-*S. aureus* nasal carriage outcomes, the association between nasal carriage outcome and SSTI was also consistently attenuated. In these regression models, we observed consistently stronger associations between OF anti-ClfA and anti-SCIN IgA antibody levels and self-reported SSTI than for *S. aureus* nasal carriage outcomes. For example, every $\log_{10}$ increase in OF anti-ClfA IgA antibody level was associated with 4.17 (95% CI:1.44, 12.1) times increased prevalence of self-reported SSTI in the model that also included *S. aureus* nasal carriage as a covariate. In the same

regression model, each $\log_{10}$ increase in OF anti-SCIN IgA antibody level was associated with 0.19 (95% CI: 0.05, .666) times the prevalence of self-reported SSTI. There was no indication of an association between OF anti-AT (neither IgA nor IgG) and self-reported SSTI. These strong associations (positive for anti-ClfA and negative for anti-SCIN) were consistent for each of the other *S. aureus* nasal carriage outcomes evaluated (*S. aureus,* MDRSA, tet[R]-*S. aureus*, LA- *S. aureus*).

DISCUSSION

We developed a multiplex immunoassay to measure IgA and IgG antibody responses to three *S. aureus* antigens (SCIN, ClfA, and AT) in OF collected from IHO workers and their household contacts in NC, USA. Previous studies have characterized important associations between serum IgA and IgG antibody responses against various *S. aureus* antigens and outcomes of *S. aureus* colonization and infection in diverse healthy populations,[27,102] hospitalized adults,[27,41] adults with invasive *S. aureus* infections,[27,110] dialysis patients,[27] intravenous drug-users,[42] and children colonized with *S. aureus* or presenting with *S. aureus* infection.[43] All of these studies have measured IgA and IgG antibody levels in serum. To our knowledge, no studies have attempted to measure or validate anti-*S. aureus* IgA and IgG antibody levels in OF in response to *S. aureus* exposure. While serum collection is a routine procedure in the healthcare setting, it is invasive, usually requires clinically trained personnel, and has practical constraints for studies involving repeated measures and susceptible populations, including young children. OF collection is minimally invasive, does not require clinical personnel, and can

be easily implemented in community and remote settings, such as the rural eastern NC project site of this IHO worker cohort.[105,109]

Our findings in OF are remarkably consistent with previous research aimed at understanding age- and *S. aureus* exposure-dependent IgG responses in diverse human populations.[27] Wu et al. reported lower serum anti-AT IgG antibody levels among children and significantly elevated anti-AT IgG antibody levels among adolescents and adults that was maintained throughout adulthood.[27] In the current study, the lowest OF IgA and IgG antibody levels for ClfA, SCIN, and AT were observed among children 7-17 years of age. This is consistent with an absence of frequent or accumulating exposure to *S. aureus* at younger ages. As age groups increased in our study population there was evidence of maturation of immunity to *S. aureus*.[27] We observed that OF IgA and IgG antibodies against SCIN, ClfA, and AT were significantly elevated, and remained stable, throughout adult ages (ages 19-49). Previous serum-based studies suggest that serum anti-AT IgG antibody levels wane at elderly ages,[27] but secretion of IgA into OF is not weakened with aging.[111] Our findings were consistent with these studies in that OF IgG antibodies against all *S. aureus* antigens diminished at elderly ages (ages 50-82) while OF IgA antibodies remained significantly elevated compared to children 7-17 years of age. Taken together, we observed that the development of OF IgA and IgG antibodies against SCIN, ClfA, and AT was an age- and *S. aureus* exposure-dependent process, and that OF IgA antibody levels against *S. aureus* SCIN, ClfA, and AT did not diminish significantly at elderly ages.

While multiple epidemiological studies suggest that IHO-workers experience a strong exposure to *S. aureus* through occupational exposure to swine,[10,15,17] this study

was the first to our knowledge to characterize associations between *S. aureus* nasal carriage outcomes and OF IgA and IgG antibody levels in a population of IHO-workers and their household contacts. Our results strongly suggest that IHO workers are occupationally exposed to, colonized by, and demonstrate early biologic effects of exposure to IHO-derived multidrug-resistant *S. aureus*. These antibody responses suggest an early biological effect indicative of *S. aureus* colonization rather than transient nasal contamination with or carriage of *S. aureus*.

Verkaik et al. showed a significant association between *S. aureus* nasal colonization and serum anti-ClfA IgA levels, and a strong correlation between antibody levels in serum and secretions from the nasal mucosa.[102] Considering the interconnected nature of the human mucosal immune system,[112] we hypothesized that anti-ClfA IgA and IgG antibody levels in OF would also be elevated among IHO-workers carrying *S. aureus* intranasally. We found a significant association of elevated anti-ClfA IgG antibody levels in OF when IHO-workers carried *S. aureus* in their nares compared to when they did not. When we classified *S. aureus* into subpopulations commonly contracted by IHO-workers (MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus*), IgA antibody levels against ClfA were elevated among IHO-workers carrying those strains vs. those who were not. Anti-ClfA IgA and IgG antibody levels in OF were not elevated among adult household-contacts of IHO-workers. These results are consistent with an elevated chronic exposure to *S. aureus*, and acute exposure to IHO-derived MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus,* among IHO-workers regularly exposed to swine and the IHO environment.

In our work, and the work of Wu et al. ,[27] IgG antibody levels of anti-AT were somewhat elevated among *S. aureus* nasal carriage compared to non-carriage, but the

association was not statistically significant. Previous studies have demonstrated a low level of *hla* gene expression in healthy individuals intranasally colonized with *S. aureus*, whereas increased *hla* gene expression and increased hemolytic activity have been correlated with *S. aureus* infection in both humans and animals.[113–115] Elevated *clfA* transcript levels have been reported in *S. aureus* implicated in nasal colonization of healthy human participants, suggesting a role for ClfA in nasal colonization.[115] It may be the case that anti-ClfA IgA and IgG antibodies and anti-AT IgA and IgG antibodies in OF serve as biomarkers for different stages of the infectious process, such as colonization and early biological effect vs. progression from colonization to infection.

While the presence and sequence of ClfA and AT are highly conserved in diverse *S. aureus* clinical isolates including LA-*S. aureus*,[116,117] the predominating LA-*S. aureus* lineages commonly contracted by IHO-workers[14,15,103] tend to lack the *scn* gene encoding for SCIN, which is known to play a role in human immune evasion.[55] We thus probed OF samples for anti-SCIN IgA and IgG antibody levels, hypothesizing that anti-SCIN IgA and IgG antibody levels would not be elevated among IHO-worker OF samples in response to *S. aureus* exposure. When analyzing IHO-workers and adults separately, we found a strong association of elevated OF anti-SCIN IgA and IgG antibody levels among *S. aureus* nasal carriage events compared to non-carriage events for adults household members only. This observation is consistent with a lack of the *scn* gene in *S. aureus* from swine and the IHO-environment,[9,14,15,17] but an almost ubiquitous presence of the *scn* gene in *S. aureus* contracted from other non-livestock-settings – e.g., the communit and hospitals.[118–120]

To determine the contribution of each *S. aureus* subpopulation to the overall OF IgA and IgG signals against *S. aureus,* we conducted three sensitivity analyses (Table 3S.3). In the first, we excluded all MDRSA observations, in the second we excluded all tet[R]-*S. aureus* observations, and in the third we excluded all LA-*S. aureus* observations. The significant association of elevated OF anti-ClfA IgG antibody levels among IHO-workers positive vs. negative for *S. aureus* nasal carriage was attenuated when either MDRSA or tet[R]-*S. aureus* observations were excluded (Table 3S.3). Thus, we believe that persistent exposure to MDRSA and tet[R]-*S. aureus* may be driving, or largely contributing to, the observed elevated OF anti-ClfA IgG antibody levels among IHO-workers in response to *S. aureus* exposure. This is consistent with previously reported occupational exposure to IHO-derived MDRSA and tet[R]-*S. aureus* experienced by IHO-workers,[14] and indicative of a heightened exposure pressure for these strains within the IHO environment.

In our previous work, we demonstrated that frequency of PPE use in IHOs was associated with *S. aureus* exposure, and that facemask use protects IHO-workers and their household contacts from IHO-derived *S. aureus* nasal carriage outcomes.[14] Thus, facemasks appear to be a protective physical barrier between *S. aureus* nasal carriage and IHO-derived *S. aureus* exposures. Congruent with these previous observations, we found that OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels were elevated among *S. aureus* carriage vs. non-carriage events only among IHO-workers who reported never wearing a facemask. This suggests that facemasks can protect IHO-workers from exposure to IHO-derived *S. aureus*, but also might mitigate early biological effects in response to IHO-derived *S. aureus* exposure. Because of the limited number of

observations in each facemask use stratum this finding should be interpreted with caution. One puzzling association with respect to facemask use was that of elevated anti-SCIN IgA and IgG antibodies among IHO workers who never used a facemask and carried *S. aureus* intranasally, particularly because the prevailing notion is that IHO workers are largely exposed to *S. aureus* lacking the *scn* gene. SCIN is a highly immunogenic *S. aureus* protein that is only known to function against the human immune system.[55,99] The *scn* and other IEC genes are encoded on a mobile genetic element called the Sa3 prophage,[55] and many factors, including exposure to commercial biocides, may induce the transfer and integration of this prophage.[121] Due to biweekly sampling, we do not know how long a particular strain of *S. aureus* inhabited IHO workers' nares and it may be the case that IHO-derived *S. aureus* rapidly integrates and expresses IEC genes when encountering a host jump from pig to the human nasal mucosa.

Previous studies of the same cohort established that IHO workers who carried *S. aureus,* MDRSA of LA-*S. aureus* were significantly more likely to report a recent SSTI compared to non-carriers.[15] Prior work has shown that *S. aureus* nasal carriage was the strongest outcome associated with self-reported SSTI (PR = 4.5; 95% CI: 1.4, 14.9).[15] When the *S.* aureus nasal carriage variable was included in a regression model that also included anti-SCIN, anti-ClfA, and anti-AT IgA antibody levels, the association between *S. aureus* nasal carriage and self-reported SSTI was attenuated (PR = 1.8; 95% CI: 0.69, 4.4), whereas anti-ClfA and anti-SCIN IgA antibody levels were strongly associated with self-reported SSTI (anti-ClfA PR = 4.2, 95% CI: 1.4, 12.1; anti-SCIN PR = 0.19, 95% CI: 0.05, 0.67). This observation was consistent when any *S. aureus* nasal carriage outcome that had been previously established as a biomarker of SSTI risk in this cohort

(i.e., MDRSA, tet[R]-*S. aureus*, or LA-*S. aureus* nasal carriage) was included in a regression model along with anti-SCIN, anti-ClfA, and anti-AT IgA antibody levels. These findings suggest that anti-ClfA and anti-SCIN IgA antibody levels are more strongly associated with self-reported SSTI among IHO workers than *S. aureus* nasal carriage outcomes are. Thus, OF anti-SCIN and anti-ClfA IgA antibody levels may serve as non-invasive and rapid biomarkers of progression from *S. aureus* colonization to SSTI among IHO workers. While increasing OF anti-ClfA IgA antibody levels were associated with an increased prevalence of reporting a recent SSTI, increasing OF anti-SCIN IgA antibody levels were associated with a decreased prevalence of reporting a recent SSTI. This is consistent with a predominance of *scn*-negative *S. aureus* being contracted by IHO workers and suggests that *S. aureus* lacking the *scn* genes may indeed have the capacity to increase SSTI risk. It was unexpected that IgA antibody levels were so strongly and consistently associated with self-reported SSTI, considering that previous literature has consistently supported a relationship between serum IgG antibody levels and risk of SSTI.[27] However, given the lack of any prior knowledge on antigen-specific antibody-based OF biomarkers of *S. aureus* SSTI among IHO workers and their household contacts, the results provided in this study warrant further evaluation in terms of their consistency and repeatability.

This study has several limitations. First, we do not have matched OF and serum samples from each participant at sampling each time point. Typically, matched OF and serum samples are used to validate and optimize OF based immunological biomarkers and immunoassays.[122] No blood samples were collected from the cohort of IHO-workers and their household contacts, which is why we chose to compare age- and exposure-

related patterns in OF IgA and IgG levels from our study to previously published literature on age- and *S. aureus* exposure-dependent serum IgA and IgG levels. Future studies should collect matched OF and serum samples in a prospective manner and evaluate assay performance characteristics of OF using serum IgA and IgG antibody levels as the gold standard reference method. Second, we probed OF samples for antigen-specific IgA and IgG antibodies against only three *S. aureus* antigens. Although we based our antigen selection on prior research aimed at understanding *S. aureus* serology and vaccine development, this study only scratched the surface of candidate OF-based antibody biomarkers of *S. aureus* exposure, colonization, and infection. The *S. aureus* genome encodes for over 2700 proteins,[40] and it is likely that additional antigen-specific antibodies may serve as unique biomarkers of *S. aureus* exposure in this population. Future studies aimed at developing OF-based antibody biomarkers of *S. aureus* exposure, colonization, and infection should multiplex using an expanded list of antigen-specific IgA and IgG antibodies against representative *S. aureus* proteins, similar to the work of Verkaik et al. in serum.[99] Third, at baseline, a total of only 12 individuals reported experiencing an SSTI in the past 3 months. Six of these individuals were IHO workers, and the remaining six were children. Due to the limited number of self-reported SSTIs in this cohort, we were unable to fully interrogate OF-based antibody biomarkers of *S. aureus* SSTI. Lastly, because of the limited number of times points per participant included in this study, we were only able to provide a population-average level effect-estimate on the repeated measures association between *S. aureus* exposure and IgA and IgG antibody levels against SCIN, ClfA, and AT in OF. Including additional time points would enable the use of statistical methods for longitudinal data analysis, and the

estimation of transient / time-varying within-person *S. aureus* exposure and OF-based antibody responses. While a population level effect is informative, a within-person effect may be more relevant for translating these findings into clinical or community based diagnostic tools.

CONCLUSIONS

Our exploration of OF IgA and IgG against the *S. aureus* antigens SCIN, ClfA, and AT among IHO-workers and their household contacts resulted in three major findings. First, the maturation OF anti-ClfA and anti-AT IgA and IgG antibody responses was an age-dependent process which is consistent with previously published age-dependent maturation of serum anti-ClfA and anti-AT IgA and IgG antibodies. While future studies should validate anti-*S. aureus* antibody levels in OF compared to those in matched serum samples, we believe these results show promise for the future development and application of OF based antibody-based biomarkers in studying *S. aureus* exposures and outcomes among IHO workers. Second, consistent with epidemiological studies, patterns of elevated OF anti-ClfA antibodies among IHO-workers suggested that IHO-workers experience unique occupational exposure pressure to *S. aureus*, including IHO-derived subpopulations of *S. aureus*. Third, also consistent with epidemiological studies, facemask use appears to provide protection against *S. aureus* exposure as noted by the attenuation of the association between *S. aureus* nasal carriage outcomes and OF anti-SCIN, anti-ClfA, and anti-AT antibody levels. Taken together, this study developed an OF-based *S. aureus* multiplex immunoassay that can be employed in repeated-measures studies in occupational and community settings, and determined that OF from IHO-workers who are occupationally exposed to IHO-derived

S. aureus, particularly MDRSA, tet[R]-*S. aureus*, and LA-*S. aureus*, displayed elevated IgA and IgG antibodies against *S. aureus*-specific antigens. OF antibodies against ClfA may serve as a promising biomarker of occupational exposure to and infection with *S. aureus* among IHO-workers.

	Visit 0 N=170	Visit 1 N=171	Visit 8 N=155	Total N=496
	n (%)	n (%)	n (%)	n (%)
Type				
Worker	85 (50)	87 (50.9)	83 (53.6)	255 (51.4)
Adult	20 (11.8)	26 (152)	21 (13.6)	67 (13.5)
Child	50 (29.4)	49 (28.7)	50 (32.3)	149 (30)
Missing	15 (8.82)	9 (5.26)	1 (0.65)	25 (5.04)
Age				
Ages 7-18	49 (28.8)	49 (28.7)	50 (32.3)	148 (29.8)
Ages 19-29	25 (14.7)	27 (15.8)	24 (15.5)	76 (15.3)
Ages 30-39	42 (24.7)	43 (25.2)	42 (27.1)	127 (25.6)
Ages 40-49	18 (10.6)	20 (11.7)	18 (11.6)	56 (11.3)
Ages 50-82	15 (8.82)	17 (9.94)	15 (9.68)	47 (9.48)
Missing	21 (12.35)	15(8.77)	6 (14.3)	42 (8.47)
***S. aureus* carriage**				
Non-carrier	87 (51.2)	83 (48.5)	84 (54.2)	254 (51.2)
Any *S. aureus*	68 (40)	78 (45.6)	70 (45.2)	216 (43.6)
MDRSA	25 (14.7)	25 (14.6)	21 (13.6)	71 (14.3)
tet[R]-*S. aureus*	21 (12.4)	23 (13.5)	19 (12.3)	63 (12.7)
LA-*S. aureus*	28 (16.5)	36 (21)	30 (19.4)	94 (19)
Missing	15 (8.82)	10 (5.85)	1 (0.65)	26 (5.24)
Facemask use				
Never	18 (21.2)	17 (19.5)	18 (21.7)	53 (20.8)
Sometimes	37 (43.5)	35 (40.2)	37 (44.6)	109 (42.8)
Always	29 (34.1)	32 (36.8)	27 (32.5)	88 (34.5)
Missing	1 (1.18)	3 (3.45)	1 (1.20)	36 (7.26)

Table 3.1. Participant and *S. aureus* isolate characteristics

Table 3.2. Nasal carriage of *S. aureus* subpopulations and OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels. Log transformed MFI values for nasal carriage of *S. aureus* subpopulation were compared to the non-carriage reference group for OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibodies. Beta coefficients and 95% confidence intervals were estimated using generalized linear models adjusted for age and within participant clustering. **Bolded*** values represent a significant trend toward elevated antibody levels in the *S. aureus* subpopulation group compared to the referent non-carriage group, using a generalized linear model, adjusted for age and within participant level clustering. *Note.* MDRSA = multidrug-resistant *S. aureus* ; tet[R]-*S. aureus* = tetracycline-resistant *S. aureus;* LA-*S. aureus* = livestock-associated *S. aureus*.

OF Antibody	Carriage type	Workers and Adults			
		N	Salivary IgA Beta coefficient (95% CI)	N	Salivary IgG Beta coefficient (95% CI)
anti-SCIN	Non-carriage	170	REF	171	REF
	Any *S. aureus* carriage	139	0.049 (-.075, .172)	137	0.058 (-.063, .178)
	MDRSA carriage	61	**0.180 (.042, .318)***	60	0.136 (-.015, .288)
	tet[R]-*S. aureus* carriage	59	0.098 (-.064, .261)	58	0.136 (-.035, .306)
	LA-*S. aureus* carriage	81	0.072 (-.073, .218)	79	0.077 (-.068, .222)
anti-ClfA	Non-carriage	170	REF	171	REF
	Any *S. aureus* carriage	139	0.109 (-0.028, 0.246)	137	0.131 (-0.0004, 0.262)
	MDRSA carriage	61	**0.281 (0.099, 0.463)***	60	0.158 (-0.005, 0.323)
	tet[R]-*S. aureus* carriage	59	0.192 (-0.011, 0.396)	58	**0.198 (0.020, 0.376)***
	LA-*S. aureus* carriage	81	0.154 (-0.023, 0.332)	79	0.140 (-0.014, 0.293)
anti-AT	Non-carriage	170	REF	171	REF
	Any *S. aureus* carriage	139	0.063 (-0.030, 0.155)	137	0.105 (-0.007, 0.217)
	MDRSA carriage	61	0.043 (-0.058, 0.144)	60	0.134 (-0.019, 0.287)
	tet[R]-*S. aureus* carriage	59	0.106 (-0.000, 0.212)	58	0.151 (-0.011, 0.312)
	LA-*S. aureus* carriage	81	0.089 (-0.004, 0.181)	79	0.093 (-0.041, 0.227)

OF Antibody	Carriage type	Workers only			
		N	Salivary IgA Beta coefficient (95% CI)	N	Salivary IgG Beta coefficient (95% CI)
anti-SCIN	Non-carriage	133	REF	134	REF
	Any *S. aureus* carriage	117	0.031 (-.111, .172)	114	0.044 (-.089, .177)
	MDRSA carriage	52	0.143 (-.017, .302)	50	0.118 (-.056, .292)
	tet[R]-*S. aureus* carriage	49	0.059 (-.131, .248)	47	0.099 (-.095, .293)
	LA-*S. aureus* carriage	69	0.041 (-.126, .208)	66	0.037 (-.123, .196)
anti-ClfA	Non-carriage	133	REF	134	REF
	Any *S. aureus* carriage	117	0.131 (-0.016, 0.279)	114	**0.156 (.014, .297)***
	MDRSA carriage	52	**0.278 (0.077, 0.479)***	50	**0.191 (.003, .378)***
	tet[R]-*S. aureus* carriage	49	0.203 (-0.016, 0.424)	47	0.185 (-.016, .000)
	LA-*S. aureus* carriage	69	0.169 (-0.023, 0.361)	66	0.126 (-.041, .294)
anti-AT	Non-carriage	133	REF	134	REF
	Any *S. aureus* carriage	117	0.040 (-0.063, 0.142)	114	0.101 (-0.022, 0.225)
	MDRSA carriage	52	0.017 (-0.093, 0.127)	50	0.133 (-0.037, 0.303)
	tet[R]-*S. aureus* carriage	49	0.066 (-0.040, 0.172)	47	0.149 (-0.034, 0.332)
	LA-*S. aureus* carriage	69	0.051 (-0.044, 0.145)	66	0.081 (--0.065, 0.228)

OF Antibody	Carriage type	Adults only			
		N	Salivary IgA Beta coefficient (95% CI)	N	Salivary IgG Beta coefficient (95% CI)
anti-SCIN	Non-carriage	37	REF	37	REF
	Any *S. aureus* carriage	22	0.116 (-.130, .363)	23	0.130 (-.136, .396)
	MDRSA carriage	9	**0.349 (.157, .541)***	10	0.211 (-.027, .449)
	tet[R]-*S. aureus* carriage	10	**0.269 (.028, .510)***	11	**0.291 (.026, .556)***
	LA-*S. aureus* carriage	12	0.206 (-.025, .438)	13	**0.271 (.009, .532)***
anti-ClfA	Non-carriage	37	REF	37	REF
	Any *S. aureus* carriage	22	-0.013 (-0.384, 0.358)	23	0.046 (-0.287, 0.379)
	MDRSA carriage	9	0.274 (-0.146, 0.694)	10	0.036 (-0.274, 0.345)
	tet[R]-*S. aureus* carriage	10	0.126 (-0.378, 0.630)	11	0.274 (-0.041, 0.589)
	LA-*S. aureus* carriage	12	0.048 (-0.408, 0.504)	13	0.251 (-0.060, 0.563)
anti-AT	Non-carriage	37	REF	37	REF
	Any *S. aureus* carriage	22	0.137 (-0.085, 0.360)	23	0.134 (-0.130, 0.397)
	MDRSA carriage	9	0.124 (-0.134, 0.383)	10	0.147 (-0.214, 0.507)
	tet[R]-*S. aureus* carriage	10	0.264 (-0.090, 0.618)	11	0.163 (-0.174, 0.500)
	LA-*S. aureus* carriage	12	0.243 (-0.064, 0.550)	13	0.159 (-0.163, 0.481)

Table 3.3. Modification of the association between *S. aureus*, MDRSA, tet[R]-S. aureus, and LA-*S. aureus* carriage and OF anti-SCIN, anti-ClfA, and anti-AT antibody levels by self-reported facemask use among IHO-workers. Log transformed MFI values for nasal carriage of *S. aureus* subpopulation were compared to the non-carriage referent group for OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibodies among IHO-workers, stratified by self-reported facemask use (never, sometimes, or always). Beta coefficients and 95% confidence intervals were estimated using generalized linear models adjusted for age and within participant clustering.. **Bolded*** values represent a significant trend toward elevated antibody levels in the *S. aureus* subpopulation group compared to the referent non-carriage group, using a generalized lineage model adjusted for age and within participant clustering. *Note.* MDRSA = multidrug-resistant *S. aureus* ; tet[R]-*S. aureus* = tetracycline-resistant *S. aureus;* LA-*S. aureus* = livestock-associated *S. aureus.*

		anti-SCIN			
		Salivary IgA		Salivary IgG	
		Beta coefficient		Beta coefficient	
		N	(95% CI)	N	(95% CI)
***S. aureus* carriage**					
Never	Non-colonized	28	REF	28	REF
	S. aureus colonized	25	-0.005 (-0.220, 0.210)	25	0.211 (-0.018, 0.439)
Sometimes	Non-carrier	59	REF	59	REF
	S. aureus colonized	50	-0.058 (-0.274, 0.160)	49	0.016 (-0.221, 0.254)
Always	Non-carrier	44	REF	45	REF
	S. aureus colonized	39	0.149 (-0.096, 0.395)	38	-0.017 (-0.209, 0.174)
MDRSA carriage					
Never	Non-carrier	28	REF	28	REF
	MDRSA colonized	16	**0.221 (0.014, 0.428)***	16	**0.376 (0.098, 0.653)***
Somtimes	Non-carrier	59	REF	59	REF
	MDRSA colonized	26	0.053 (-0.183, 0.289)	25	0.002 (-0.291, 0.295)
Always	Non-carrier	44	REF	45	REF
	MDRSA colonized	8	0.053 (-0.232, 0.338)	8	-0.005 (-0.176, 0.167)
Tet[R]-*S. aureus* carriage					
Never	Non-carrier	28	REF	28	REF
	tet[R]-*S. aureus* colonized	13	**0.176 (0.005, 0.347)***	13	**0.368 (0.070, 0.665)***
Sometimes	Non-carrier	59	REF	59	REF
	tet[R]-*S. aureus* colonized	24	0.056 (-0.199, 0.311)	23	-0.008 (-0.022, 0.006)
Always	Non-carrier	44	REF	45	REF
	tet[R]-*S. aureus* colonized	10	-0.224 (-0.536, 0.087)	10	-0.001 (-0.009, 0.006)
LA-*S. aureus* carriage					
Never	Non-carrier	28	REF	28	REF
	LA-*S. aureus* colonized	18	0.139 (-0.042, 0.319)	18	0.256 (-0.004, 0.516)
Sometimes	Non-carrier	59	REF	59	REF
	LA-*S. aureus* colonized	34	-0.010 (-0.246, 0.227)	33	-0.005 (-0.260, 0.250)
Always	Non-carrier	44	REF	45	REF
	LA-*S. aureus* colonized	15	-0.062 (-0.386, 0.263)	14	-0.142 (-0.349, 0.066)

		anti-ClfA			
		Salivary IgA		Salivary IgG	
		Beta coefficient		Beta coefficient	
		N	(95% CI)	N	(95% CI)
***S. aureus* carriage**					
Never	Non-colonized	28	REF	28	REF
	S. aureus colonized	25	0.094 (-0.204, 0.392)	25	**0.281 (0.068, 0.493)***
Sometimes	Non-carrier	59	REF	59	REF
	S. aureus colonized	50	0.055 (-0.168, 0.278)	49	0.146 (-0.081, 0.372)
Always	Non-carrier	44	REF	45	REF
	S. aureus colonized	39	0.233 (-0.031, 0.496)	38	0.050 (-0.191, 0.291)
MDRSA carriage					
Never	Non-carrier	28	REF	28	REF
	MDRSA colonized	16	**0.473 (0.218, 0.728)***	16	**0.437 (0.090, 0.785)***
Somtimes	Non-carrier	59	REF	59	REF
	MDRSA colonized	26	0.124 (-0.177, 0.425)	25	0.078 (-0.165, 0.319)
Always	Non-carrier	44	REF	45	REF
	MDRSA colonized	8	0.139 (-0.141, 0.419)	8	-0.036 (-0.306, 0.235)
Tet[R]-*S. aureus* carriage					
Never	Non-carrier	28	REF	28	REF
	tet[R]-*S. aureus* colonized	13	**0.418 (0.156, 0.681)***	13	**0.513 (0.180, 0.845)***
Sometimes	Non-carrier	59	REF	59	REF
	tet[R]-*S. aureus* colonized	24	0.115 (-0.192, 0.422)	23	0.104 (-0.167, 0.376)
Always	Non-carrier	44	REF	45	REF
	tet[R]-*S. aureus* colonized	10	-0.026 (-0.402, 0.351)	10	-0.124 (-0.351, 0.102)
LA-*S. aureus* carriage					
Never	Non-carrier	28	REF	28	REF
	LA-*S. aureus* colonized	18	**0.356 (0.114, 0.599)***	18	**0.317 (0.046, 0.589)***
Sometimes	Non-carrier	59	REF	59	REF
	LA-*S. aureus* colonized	34	0.063 (-0.214, 0.340)	33	0.120 (-0.107, 0.347)
Always	Non-carrier	44	REF	45	REF
	LA-*S. aureus* colonized	15	0.062 (-0.256, 0.379)	14	-0.172 (-0.388, 0.044)

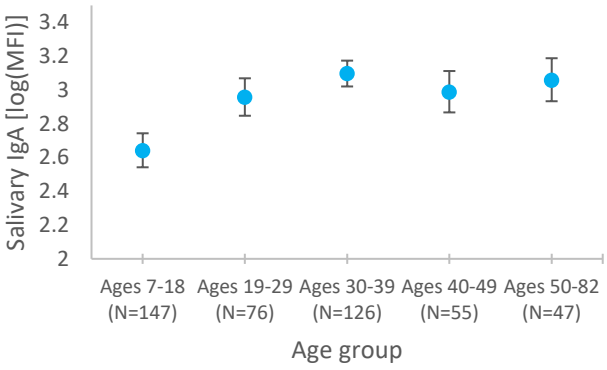
		anti-AT			
			Salivary IgA		**Salivary IgG**
			Beta coefficient		**Beta coefficient**
		N	**(95% CI)**	N	**(95% CI)**
***S. aureus* carriage**					
Never	Non-colonized	28	REF	28	REF
	S. aureus colonized	25	-0.071 (-0.224, 0.082)	25	0.128 (-0.062, 0.319)
Sometimes	Non-carrier	59	REF	59	REF
	S. aureus colonized	50	0.003 (-0.128, 0.133)	49	0.091 (-0.115, 0.297)
Always	Non-carrier	44	REF	45	REF
	S. aureus colonized	39	0.138 (-0.118, 0.393)	38	0.049(-0.153, 0.250)
MDRSA carriage					
Never	Non-carrier	28	REF	28	REF
	MDRSA colonized	16	0.064 (-0.107, 0.235)	16	0.181 (-0.048, 0.411)
Somtimes	Non-carrier	59	REF	59	REF
	MDRSA colonized	26	-0.053 (-0.182, 0.075)	25	0.069 (-0.195, 0.333)
Always	Non-carrier	44	REF	45	REF
	MDRSA colonized	8	0.042 (-0.374, 0.458)	8	0.054 (-0.280, 0.389)
Tet[R]-*S. aureus* carriage					
Never	Non-carrier	28	REF	28	REF
	tet[R]-*S. aureus* colonized	13	0.060 (-0.098, 0.218)	13	**0.290 (0.045, 0.536)***
Sometimes	Non-carrier	59	REF	59	REF
	tet[R]-*S. aureus* colonized	24	-0.019 (-0.149, 0.111)	23	0.088 (-0.195, 0.370)
Always	Non-carrier	44	REF	45	REF
	tet[R]-*S. aureus* colonized	10	0.210 (-0.093, 0.513)	10	-0.024 (-0.385, 0.338)
LA-*S. aureus* carriage					
Never	Non-carrier	28	REF	28	REF
	LA-*S. aureus* colonized	18	0.037 (-0.103, 0.178)	18	0.158 (-0.041, 0.358)
Sometimes	Non-carrier	59	REF	59	REF
	LA-*S. aureus* colonized	34	-0.015 (-0.128, 0.098)	33	0.042 (-0.175, 0.258)
Always	Non-carrier	44	REF	45	REF
	LA-*S. aureus* colonized	15	0.156 (-0.116, 0.429)	14	-0.048 (-0.317, 0.222)

	Skin and soft tissue infection outcome PR (95% CI)							
	IgA				IgG			
Covariate	**Model 1**	**Model 2**	**Model 3**	**Model 4**	**Model 1**	**Model 2**	**Model 3**	**Model 4**
anti-SCIN	**0.19 , (0.05, .666)***	**0.17 (0.04, 0.75)***	**0.18 (0.04, 0.69)***	**0.17 (0.04, 0.81)***	1.02 (0.29, 3.61)	0.90 (0.25, 3.28)	0.94 (0.26, 3.44)	1.10 (.338, 3.61)
anti-ClfA	**4.16 (1.44, 12.1)***	**4.57 (1.39, 15.05)***	**4.48 (1.52, 13.25)***	**4.57 (1.33, 15.68)***	1.04 (0.25, 4025)	1.02 (0.21, 4.98)	1.15 (0.26, 5.19)	.933 (0.21, 4.14)
anti-AT	0.79 (0.15, 4.24)	0.84 (0.13, 5.38)	0.81 (0.13, 5.02)	0.78 (0.13, 4.61)	0.69 (0.23, 2.07)	0.73 (0.24, 2.22)	0.68 (0.21, 2.14)	0.67 (0.21, 2.09)
S. aureus	1.75 (0.69, 4.45)	-	-	-	1.88 (0.71, 4.95)	-	-	-
MDRSA	-	2.15 (0.78, 5.91)	-	-	-	2.31 (0.77, 6.88)	-	-
Tet[R]-*S. aureus*	-	-	1.13 (0.39, 3.28)	-	-	-	1.14 (0.39, 3.34)	-
LA-*S. aureus*	-	-	-	2.35 (0.89, 6.20)	-	-	-	**2.63 (1.03, 6.69)***

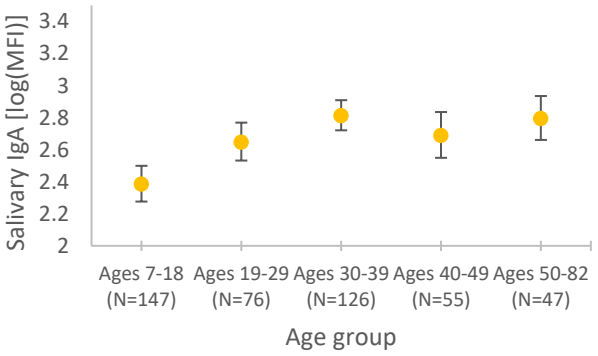
Table 3.4. OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels and self-reported SSTI. The relationship between prevalence of self-reported SSTI and $\log_{10}$ transformed OF IgA and IgG anti-SCIN, anti-ClfA, and anti-AT antibody levels was examined. Prevalence ratios and 95% confidence intervals were estimated using generalized linear models adjusted for age and within participant clustering. For both IgA and IgG antibody targets, model 1 included the *S. aureus* nasal carriage variable as a covariate, model 2 included the MDRSA nasal carriage variable as a covariate, model 3 included the tet[R]-*S. aureus* nasal carriage variable as a covariate, and model 4 included the LA-*S. aureus* nasal carriage variable as a covariate. **Bolded*** values represent a prevalence ratio that is significantly different from 1. *Note.* MDRSA = multidrug-resistant *S. aureus* ; tet[R]-*S. aureus* = tetracycline-resistant *S. aureus;* LA-*S. aureus* = livestock-associated *S. aureus;* SCIN = staphylococcal complement inhibitor; ClfA = clumping factor A; AT = alpha toxin.

<u>Figure 3.1.</u> Age and OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels. Salivary IgA and IgG antibody levels in IHO-workers and their household contacts by different age groups. Geometric mean values and 95% confidence intervals are shown for A). Salivary anti-SCIN IgA, B). Salivary anti-ClfA IgA, C). Salivary anti-AT IgA, D). Salivary anti-SCIN IgG, E). Salivary anti-ClfA IgG, and F). Salivary anti-AT IgG.

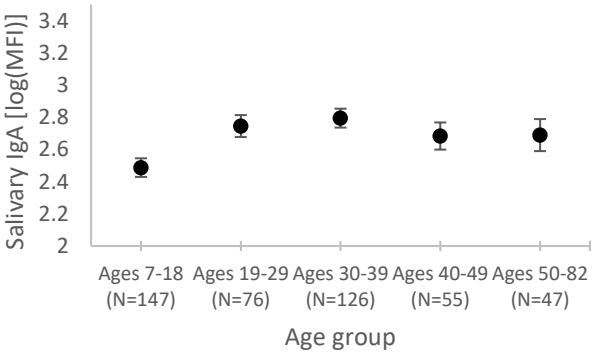
A). Salivary anti-SCIN IgA antibody levels by age group



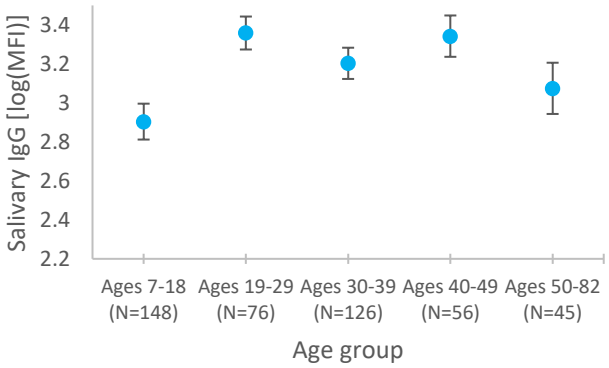
B). Salivary anti-ClfA IgA antibody levels by age group



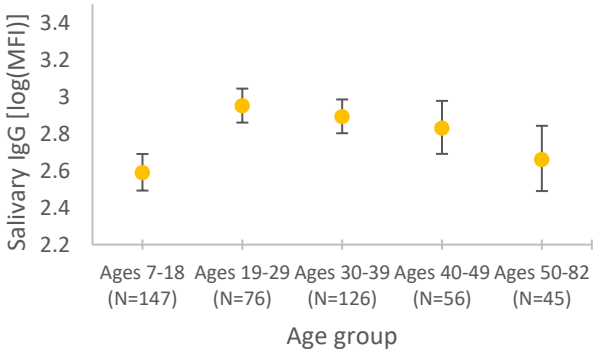
C). Salivary anti-AT IgA antibody levels by age group



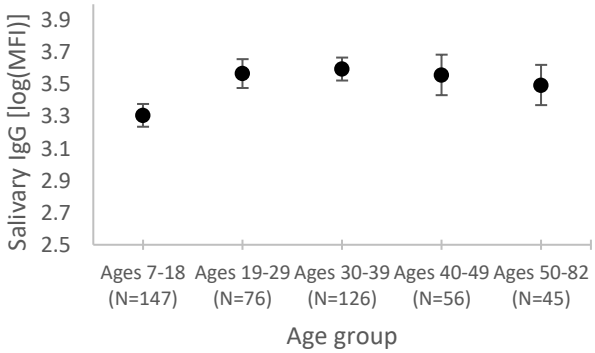
D). Salivary anti-SCIN IgG antibody levels by age group



E). Salivary anti-ClfA IgG antibody levels by age group



F). Salivary anti-AT IgG antibody levels by age group



SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Antimicrobial Agent	Susceptible (µg/mL)	Intermediate (ug/mL)	Resistant (µg/mL)
Penicillin	</=0.2	N/A	>/=.25
Erythromycin	</=0.5	1.0-4.0	>/= 8
Clindamycin	0.5	1.0-2.0	>/= 4.0
Moxifloxacin	</=0.5	1	>/= 2.0
Tetracycline	</=4	8	>/=16.0
Trimethoprim/sulfamethoxazole	</=2/38	N/A	>/=4/76
Gentamycin	</=4	8	>/=16
Cefoxitin	</=4	N/A	>/=4
Oxacillin	</= 0.25	N/A	>/=0.5
Quinupristin/dalfopristin	</=1	2	>/=4
Minocycline	</=4	8	>/=16.0
Nitrofurantoin	</=32	64	>/=16
Rifampin	</=1	2	>/=4
Linezolid	</=4	N/A	>/=8
Daptomycin	</=1	N/A	N/A
Vancomycin	</=2	4.0-8.0	>/= 16

Table 3S.1. Minimum inhibitory concentration (MIC) cut-off values for antibiotic susceptibility testing

anti-SCIN IgA				**anti-SCIN IgG**			
		Lower	Upper			Lower	Upper
Age bin	log(MFI)	CI	CI	Age bin	log(MFI)	CI	CI
Ages 7-18 (N=147)	2.64	2.54	2.74	Ages 7-18 (N=148)	2.90	2.81	2.99
Ages 19-29 (N=76)	**2.95**	**2.85**	**3.06**	Ages 19-29 (N=76)	**3.35**	**3.27**	**3.44**
Ages 30-39 (N=126)	**3.09**	**3.02**	**3.17**	Ages 30-39 (N=126)	**3.20**	**3.12**	**3.28**
Ages 40-49 (N=55)	**2.98**	**2.86**	**3.11**	Ages 40-49 (N=56)	**3.34**	**3.23**	**3.44**
Ages 50-82 (N=47)	**3.05**	**2.93**	**3.18**	Ages 50-82 (N=45)	3.07	2.94	3.20
anti-ClfA IgA				**anti-ClfA IgG**			
		Lower	Upper			Lower	Upper
Age bin	log(MFI)	CI	CI	Age bin	log(MFI)	CI	CI
Ages 7-18 (N=147)	2.38	2.27	2.49	Ages 7-18 (N=147)	2.58	2.49	2.69
Ages 19-29 (N=76)	2.64	2.53	2.76	Ages 19-29 (N=76)	**2.95**	**2.85**	**3.04**
Ages 30-39 (N=126)	**2.81**	**2.72**	**2.90**	Ages 30-39 (N=126)	**2.89**	**2.80**	**2.98**
Ages 40-49 (N=55)	**2.68**	**2.54**	**2.83**	Ages 40-49 (N=56)	2.83	2.69	2.97
Ages 50-82 (N=47)	**2.79**	**2.66**	**2.93**	Ages 50-82 (N=45)	2.66	2.48	2.84
anti-AT IgA				**anti-AT IgG**			
		Lower	Upper			Lower	Upper
Age bin	log(MFI)	CI	CI	Age bin	log(MFI)	CI	CI
Ages 7-18 (N=147)	2.48	2.42	2.54	Ages 7-18 (N=147)	3.30	3.23	3.37
Ages 19-29 (N=76)	**2.74**	**2.67**	**2.81**	Ages 19-29 (N=76)	**3.56**	**3.47**	**3.65**
Ages 30-39 (N=126)	**2.79**	**2.73**	**2.85**	Ages 30-39 (N=126)	**3.59**	**3.52**	**3.66**
Ages 40-49 (N=55)	**2.68**	**2.59**	**2.76**	Ages 40-49 (N=56)	**3.55**	**3.43**	**3.68**
Ages 50-82 (N=47)	**2.68**	**2.58**	**2.78**	Ages 50-82 (N=45)	3.49	3.37	3.62

Table 3S.2. Age and OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels. The geometric mean of log transformed MFI values and 95% confidence within each age bin are shown for oral fluid anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibodies. For each IgA and IgG target, each age bin was compared to the Ages 7-18 (minor) age bin using a generalized linear model, adjusted for within participant clustering. **Bolded** values display a significant trend towards elevated MFI values compared to the referent minor group.

Table 3S.3. Nasal carriage of *S. aureus* and OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels after exclusion of IHO-derived *S. aureus* subpopulations. Beta coefficients and 95% confidence intervals were estimated using generalized linear models adjusted for age and within participant clustering. MDRSA = multidrug-resistant *S. aureus*; Tet[R]-*S. aureus* = tetracycline-resistant *S. aureus;* LA-*S. aureus* = livestock-associated *S. aureus.*

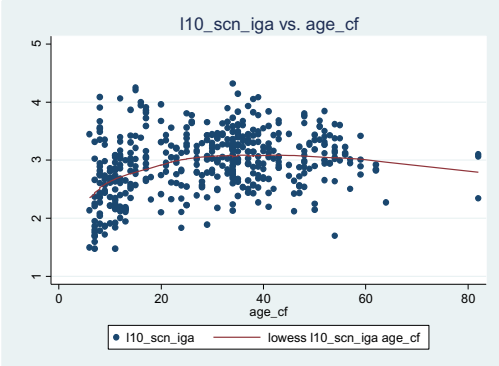
		Workers and Adults			
			Salivary IgA		Salivary IgG
		N	Beta coefficient (95% CI)	N	Beta coefficient (95% CI)
MDRSA carriage events excluded					
SCIN	Non-carrier	170	REF	171	REF
	S. aureus carriage	*78*	-.056 (-.210, .099)	77	-.004 (.148, .139)
ClfA	Non-carrier	170	REF	171	REF
	S. aureus colonized	*78*	-0.026 (-.184, .131)	77	0.109 (-.054, .272)
AT	Non-carrier	170	REF	171	REF
	S. aureus colonized	*78*	0.078 (-.044, .201)	77	.082 (-.040, .203)
Tet[R]-*S. aureus* carriage events excluded					
SCIN	Non-carrier	170	REF	171	REF
	S. aureus colonized	*80*	.012 (-.135, .158)	79	.000 (-.123, .124)
ClfA	Non-carrier	170	REF	171	REF
	S. aureus colonized	*80*	.048 (-.102, .198)	79	.081 (-.061, .224)
AT	Non-carrier	170	REF	171	REF
	S. aureus colonized	*80*	0.031 (-.091, .153)	79	.071 (-.040, .183)
LA-*S. aureus* carriage events excluded					
SCIN	Non-carrier	170	REF	171	REF
	S. aureus colonized	*58*	0.015 (-.159, .189)	58	.031 (-.114, .176)
ClfA	Non-carrier	170	REF	171	REF
	S. aureus colonized	*58*	.045 (-.131, .222)	58	.118 (-.046, .283)
AT	Non-carrier	170	REF	171	REF
	S. aureus colonized	*58*	0.027 (-.130, .183)	58	.122 (-.006, .250)

		Workers only			
		N	Salivary IgA Beta coefficient (95% CI)	N	Salivary IgG Beta coefficient (95% CI)
MDRSA carriage events excluded					
SCIN	Non-carrier	133	REF	134	REF
	S. aureus carriage	65	-0.06 (-.238, .118)	64	-.015 (-.172, .142)
ClfA	Non-carrier	133	REF	134	REF
	S. aureus colonized	65	0.013 (-.158, .183)	64	0.128 (-.045, .300)
AT	Non-carrier	133	REF	134	REF
	S. aureus colonized	65	0.058 (-.077, .192)	64	0.076 (-.057, .209)
Tet[R]-*S. aureus* carriage events excluded					
SCIN	Non-carrier	133	REF	134	REF
	S. aureus colonized	68	.011 (-.154, .175)	67	.005 (-.133, .143)
ClfA	Non-carrier	133	REF	134	REF
	S. aureus colonized	68	.079 (-.081, .239)	67	.135 (-.016, .286)
AT	Non-carrier	133	REF	134	REF
	S. aureus colonized	68	.021 (-.118, .159)	67	.068 (-.050, .186)
LA-*S. aureus* carriage events excluded					
SCIN	Non-carrier	133	REF	134	REF
	S. aureus colonized	48	.015 (-.179, .210)	48	.054 (-.113, .222)
ClfA	Non-carrier	133	REF	134	REF
	S. aureus colonized	48	.077 (-.111, .265)	48	**0.196 (.016, .377)***
AT	Non-carrier	133	REF	134	REF
	S. aureus colonized	48	.024 (-.158, .206)	48	.130 (-.009, .268)

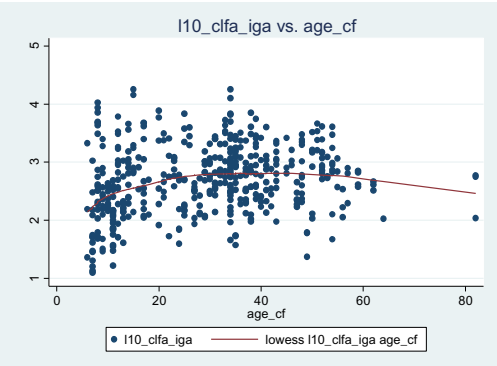
		Adults only			
		N	Salivary IgA Beta coefficient (95% CI)	N	Salivary IgG Beta coefficient (95% CI)
MDRSA carriage events excluded					
SCIN	Non-carrier	37	REF	37	REF
	S. aureus carriage	13	-0.045 (-.343, .252)	13	.067 (-.280, .415)
ClfA	Non-carrier	37	REF	37	REF
	S. aureus colonized	13	-.212 (-.567, .143)	13	.054 (-.386, .494)
AT	Non-carrier	37	REF	37	REF
	S. aureus colonized	13	0.146 (-.153, .446)	13	0.124 (-.180, .428)
Tet[R]-*S. aureus* carriage events excluded					
SCIN	Non-carrier	37	REF	37	REF
	S. aureus colonized	12	-.011 (-.344, .322)	12	-.018 (-.308, .272)
ClfA	Non-carrier	37	REF	37	REF
	S. aureus colonized	12	-.128 (-.493, .236)	12	-.164 (-.506, .178)
AT	Non-carrier	37	REF	37	REF
	S. aureus colonized	12	.032 (-.167, .230)	12	.107 (-.217, .430)
LA-*S. aureus* carriage events excluded					
SCIN	Non-carrier	37	REF	37	REF
	S. aureus colonized	10	.010 (-.368, .387)	10	-.052 (-.350, .246)
ClfA	Non-carrier	37	REF	37	REF
	S. aureus colonized	10	-.085 (-.500, .331)	10	-0.220 (-.558, .119)
AT	Non-carrier	37	REF	37	REF
	S. aureus colonized	10	.011 (-.209, .232)	10	.101 (-.245, .446)

<u>Figure 3S.1.</u> Scatter plot of age vs. OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels with lowess curves. A). Salivary anti-SCIN IgA, B). Salivary anti-ClfA IgA, C). Salivary anti-AT IgA, D). Salivary anti-SCIN IgG, E). Salivary anti-ClfA IgG, and F). Salivary anti-AT IgG.

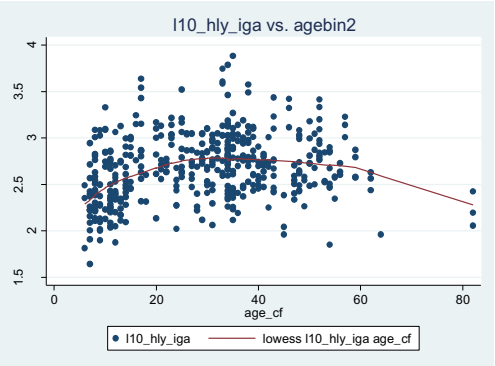
A).


l10_scn_iga vs. age_cf
age_cf
l10_scn_iga
lowess l10_scn_iga age_cf

B).


l10_clfa_iga vs. age_cf
age_cf
l10_clfa_iga
lowess l10_clfa_iga age_cf

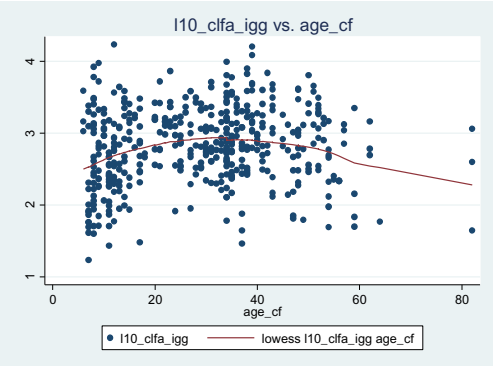
C).


l10_hly_iga vs. agebin2
age_cf
l10_hly_iga
lowess l10_hly_iga age_cf

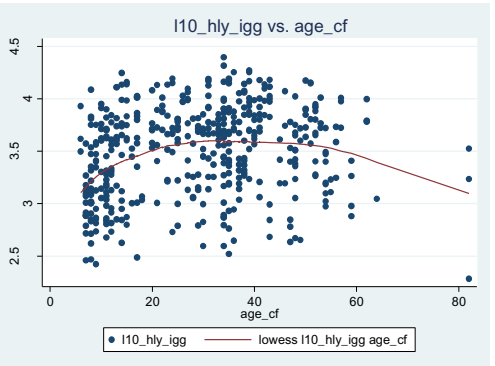
D).


l10_scn_igg vs. age_cf
age_cf
l10_scn_igg
lowess l10_scn_igg age_cf

E).


l10_clfa_igg vs. age_cf
age_cf
l10_clfa_igg
lowess l10_clfa_igg age_cf

F).


l10_hly_igg vs. age_cf
age_cf
l10_hly_igg
lowess l10_hly_igg age_cf

CHAPTER 4

Conclusions and future directions

CONCLUSIONS

The dissemination of LA-*S. aureus* from IHOs into human populations, and human infection with multidrug-resistant LA-*S. aureus*, pose urgent public health concerns. The intensive use of antimicrobials in food animal production strongly suggests that pigs raised on IHOs are the source of occupational and community exposure to multidrug-resistant LA-*S. aureus*.[44] The objectives of this dissertation were to conduct research aimed at filling knowledge gaps in our understanding of the global and regional transmission dynamics of LA-*S. aureus*, the relative pathogenicity of LA-*S. aureus* compared to epidemic SSTI CA-MRSA strains, and novel non-invasive immunological biomarkers of *S. aureus* colonization and infection among IHO workers.

The research outlined in this dissertation addressed these critical knowledge gaps. In Chapter 1, a WGS and phylogenetic-analysis was conducted on a representative subset of LA-*S. aureus* CC9 isolates collected from IHO pigs, IHO workers, IHO worker household contacts, and community residents with no known exposure to livestock, aimed to elucidate the population structure of LA-*S. aureus* CC9, and to provide evidence of transmission of LA-*S. aureus* CC9 between IHO pigs and humans in NC, USA. Chapter 1 also examined the role of acquired antimicrobial resistance in pig-human transmission of LA-*S. aureus* CC9. In Chapter 2, *in vivo* animal studies were conducted to compare the relative pathogenicity of LA-*S. aureus* strains commonly contracted by IHO workers in the USA to highly pathogenic epidemic CA-MRSA SSTI strains in a mouse model of SSTI, and the innate-immune response in LA-*S. aureus* vs. CA-MRSA infected mice. Finally, Chapter 3 of dissertation demonstrated successful development and application of an OF multiplex *S. aureus*-specific antibody-based immunoassay to

investigate biomarkers of *S. aureus* colonization and infection among IHO workers and their household contacts in NC, USA.

Research conducted in this dissertation contributed significantly to our understanding of transmission dynamics of LA-*S. aureus* between IHO pigs and humans, the relative pathogenicity of LA-*S. aureus* commonly contracted by IHO workers compared to highly pathogenic epidemic CA-MRSA SSTI strains, and biomarkers of early biological effect in response to *S. aureus* exposure among IHO workers. Taken together, the research provided in this dissertation suggested that:

1. Clonal expansion of LA-*S. aureus* CC9 in the USA is distinct from those of European and Asian LA-*S. aureus* CC9 lineages.
2. LA-*S. aureus* CC9 can be transmitted between IHO pigs and humans, and two factors—a multidrug-resistant phenotype and a significantly-increased number of acquired AMR genes—are associated with pig-human transmission clusters.
3. LA-*S. aureus* CC398 and CC9 commonly contracted by IHO workers display an equivalent or greater degree of pathogenicity compared to CA-MRSA USA300 clone, SF8300, in a mouse model of SSTI.
4. Oral fluid (OF) IgA and IgG antibodies directed against ClfA, in particular, may serve as non-invasive biomarkers of *S. aureus* colonization, and oral fluid IgA antibodies directed against ClfA and SCIN may serve as non-invasive biomarkers of *S. aureus* SSTI among IHO workers.

Prior to this dissertation, LA-*S. aureus* in the USA had largely been studied in the context of occupational exposure,[9,14,15,17] with some stakeholders asserting that community exposures are benign. This dissertation strongly suggests that LA-*S. aureus*

should be considered more than a benign exposure, and rather as a potential health hazard of broader public health concern. This dissertation is the first research study, to our knowledge, to show that multidrug-resistant LA-*S. aureus* can be transmitted between IHO pigs, IHO workers, and community residents who reported no exposure to livestock, suggesting this health hazard is not limited to the occupational setting. This is especially concerning, considering that isolates implicated in pig-human transmission were enriched with multiple determinants conferring resistance to antibiotics that are critically important for human health.[72] Contrary to previous claims that LA-*S. aureus* are less pathogenic than typical HA- and CA- *S. aureus* strains,[12,19–22] this dissertation provided *in vivo* evidence that LA-*S. aureus* are not only pathogenic, but display increased pathogenicity and fitness-for-survival compared to highly pathogenic epidemic CA-MRSA SSTI strains (i.e. USA300 clone, SF8300).[37] Finally, in contrast to previous claims suggesting that LA-*S. aureus* display a reduced capacity for human colonization,[21,22] this dissertation showed elevated IgA and IgG antibody levels against ClfA in OF collected from IHO workers positive for *S. aureus* nasal carriage outcomes, especially MDRSA and tet-[R]-*S. aureus* nasal carriers compared to non-carriers. Elevated antibody levels against ClfA is an early biological effect indicative of colonization of the nasal mucosa with *S. aureus*,[99] suggesting that nasal carriage of *S. aureus* among IHO workers is not merely transient contamination but true colonization of the nasal mucosa.

To protect the U.S. public from exposure, colonization, and infection with IHO-derived multidrug-resistant LA-*S. aureus*, this dissertation highlights the importance for future research of effective interventions that can prevent the expansion and transmission of multidrug-resistant LA-*S. aureus* in human populations. Preventative interventions

should be designed to mitigate IHO production practices that promote the expansion of multidrug-resistant LA-*S. aureus*, namely the intensive use of antibiotics. The results of this dissertation are immediately applicable to on-going community and worker health hazard concerns[63] regarding harmful IHO-related exposures, such as the acquisition of multidrug-resistant LA-*S. aureus* in NC and other regions of the USA that have a high density of IHOs. Furthermore, this dissertation provides non-invasive immunological biomarkers of *S. aureus* colonization and infection that may improve longitudinal surveillance of *S. aureus* outcomes in IHO worker populations and susceptible subgroups of children who live in the same household with IHO workers.

FUTURE RESEARCH

This dissertation is foundational to several important areas of future research. In this dissertation we rooted our high-resolution phylogenetic tree at the midpoint, and are therefore unsure if the most ancestral clade of LA-*S. aureus* CC9 is of human or animal origin. By testing multiple outgroups in the analysis provided in Chapter 1, an inference on the most ancestral clade and directionality of transmission of LA-*S. aureus* CC9 between IHO pigs and humans in could be provided. Regarding the evolutionary history of LA-*S. aureus* CC9, this is a research question of critical importance, and an important pursuit for future research. Furthermore, our collection of *S. aureus* from IHO pigs from NC, USA was limited, and a more comprehensive WGS dataset may reveal intercontinental spread and additional pig-human transmission clusters of LA-*S. aureus* CC9. Future studies aimed to understand transmission dynamics of LA-*S. aureus* between IHO pigs and humans may consider to include a well-represented collection of IHO pig LA-*S. aureus* isolates. In Chapter 2 of this dissertation, the pathogenicity of only three LA-*S. aureus* isolates was compared to the referent CA-MRSA SF8300 isolate. Previous research has established a diversity of mobile genetic elements (MGEs) carried by different LA-*S. aureus* strains.[100] Considering that MGEs of *S. aureus* can encode for important virulence factors in *S. aureus* pathogenesis, and that closely related isolates of *S. aureus* may display differences in pathogenicity, future studies should compare multiple isolates of each LA-*S. aureus* lineage to make confident lineage related conclusions about the relative pathogenicity of LA-*S. aureus*. In Chapter 3 of this dissertation, the cohort of IHO workers and their household contacts included only 12

individuals with a self-reported recent SSTI. Although this chapter began to examine relationships between OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG antibody levels and self-reported SSTI, the analysis suffered due to the small number of self-reported SSTI's. To develop biomarkers of *S. aureus* SSTI, which has applicability in occupational as well as community and hospital settings, future studies should be designed to collect matched serum and OF samples at acute and convalescent time points following culture-confirmed *S. aureus* SSTI. In future *S. aureus* antibody biomarker development studies, the list of *S. aureus* antigens should be expanded for discovery of additional antigen-specific antibody-based OF biomarkers. Furthermore, antigen-specific antibody-based OF biomarkers for *S. aureus* should be developed into point-of-use assays that could facilitate minimally invasive, rapid, and user friendly testing outputs that could be integrated into occupational health and safety and community health surveillance systems without the need for clinically trained phlebotomists or personnel.

MISCELLANEOUS METHODS AND RESULTS

Miscellaneous Methods

Illumina Sequencing datasets.

Quality of sequence data was determined based on two criteria: 1). Genome size at 25x of depth (i.e. coverage) and 2). percent of reads unclassified or classified as non-*S. aureus* (i.e. contamination). Only isolates with at least 2,000,000 bp's of length at 25x of depth, and no greater than 10% of reads classified as contaminated or unclassified, were included in the dataset for phylogenetic analysis.

Average pairwise SNP-distances.

Average pairwise SNP-distances and 95% CI were estimated for each transmission cluster and the IHO pig cluster in the high-resolution phylogeny. Average pairwise SNP-distances were calculated using the bestsnp.tsv SNP matrix output of the NASP pipeline,[48] following removal of recombinant tracks of DNA.[51]

Growth curves for SF8300, NCHW8, IHW398-1, IHW398-2, and NCHW9 isolates.

S. aureus strains were streaked onto tryptic soy agar plates and grown overnight. Single colonies were selected and grown in tryptic soy broth (TSB) at 37°C in a shaking incubator overnight, shaking at 240 rpm and then sub-cultured at a 1:50 dilution in TSB. At 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 hours, 100 µL of the sub-cultures were pipetted onto a 96-well plate and absorbance (600 nm) was read with a Synergy H1 Hybrid Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

Candidate* S. aureus *antigens with references.

An extensive literature review was conducted to determine candidate *S. aureus* antigens that show potential as biomarkers of *S. aureus* colonization and infection. The literature review focused on antigen-specific antibody-based biomarkers of *S. aureus* colonization and infection. Antigens were considered candidate biomarkers if they were being used in a vaccine currently undergoing clinical trials, if previous studies have suggested considerable immunogenicity of the antigen in humans, or if previous studies have established that the antigen can serve as a biomarker of *S. aureus* colonization or infection.

Coupling confirmation for* S. aureus *antigen xMap™ beads using pre- and post-infection mouse serum.

Five (5) unique *S. aureus* antigen-coupled bead sets were prepared initially. Briefly, Staphylococcal Complement Inhibitor (SCIN), Clumping Factor A (ClfA), Clumping Factor B (ClfB), Alpha toxin (AT), and Beta toxin (BT) proteins were each covalently coupled to a unique magnetic microparticle bead set according to . Successful coupling of each bead set was confirmed using pre-infection (day 0), acute-phase (day 14) and convalescent-phase (day 28) serum from mice intradermally inoculated with *S. aureus*. 1500 beads per bead set (SCIN, ClfA, ClfB, AT, BT) were incubated with 50ul of pre-infection, acute-phase, or convalescent-phase mouse sera, diluted 1:1000 in assay buffer (PBS with 0.05% Tween20 and 1% bovine serum albumin), for 1 hour on a plate shaker at 500 rpm. Beads were washed 3 times and then incubated with 50ul of PE-labelled anti-mouse IgG diluted 1:100 in assay buffer for 1 hour on a plate shaker at 500 rpm. Beads were washed again and then suspended in 100ul assay buffer. The fluorescence signal was measured on a Bio-Plex 200 instrument (Bio-Rad). Two (2)

blanks were included on each plate to subtract background, and ~10% of OF samples were tested in duplicate to determine intra-assay variability.

Preliminary examination of 22 oral fluid samples using a 5-plex* S. aureus *immunoassay.

22 OF samples were initially selected to probe for IgA and IgG antibodies directed against SCIN, ClfA, ClfB, AT, and BT. All OF samples were selected from biweekly visit 8. 11 were selected from persistent *S. aureus* carriers (*S. aureus* positive nasal carriage at all 8 biweekly visits), and 11 were selected from *S. aureus* non-carriers (negative for *S. aureus* nasal carriage at all 8 biweekly visits). 10ul of the oral fluid supernatant was added to 40ul of assay buffer (PBS with 0.05% Tween20 and 1% bovine serum albumin) containing 1500 beads of each bead set (SCIN, ClfA, ClfB, AT, BT) per microplate well. The plate was covered and incubated at room temperature for 1 hour on a plate shaker at 500 rpm. Beads were washed 3 times and then incubated with 50ul of PE-labelled anti-human IgA diluted 1:100 in assay buffer for 1 hour on a plate shaker at 500 rpm. Beads were washed again and then suspended in 100ul assay buffer. The fluorescence signal was measured on a Bio-Plex 200 instrument (Bio-Rad). The same process was repeated, but using PE-labelled anti-human IgG diluted 1:100 for the secondary incubation. Two (2) blanks were included on each plate to subtract background, and ~10% of OF samples were tested in duplicate to determine intra-assay variability. Average MFI, and 95% confidence intervals, were calculated for each antigen-specific antibody target stratified by *S. aureus* carrier status (persistent vs. non-carrier). The average MFI for each antigen-specific antibody target among persistent carriers was compared to that of non-carriers using a students *t* test.

Correlation between OF IgA and IgG antibody levels against SCIN, ClfA, and AT.

Spearman correlation coefficients were generated to assess the relationship between IgA and IgG levels for each *S. aureus* specific antigen target (SCIN, ClfA, AT).

Miscellaneous Results

High quality whole genome sequencing datasets were included in phylogenetic analyses.

Isolates were included in the dataset for phylogenetic analysis if they had a coverage of greater than 2,000,000 bp at 25x of depth, and minimal percentage of contamination or unclassified reads. 49 MLST confirmed LA-*S. aureus* CC9 genomes were sequenced at an average depth of 65.76x (SD = 27.66), using the 2,815,299 base IHOW6 chromosome as a reference (Table 1M.1). Less than 10% of reads were unclassified for all isolates included in the dataset (Table 1M.1).

Average pairwise SNP-distances for transmission cluster and IHO pig cluster isolates in the high-resolution phylogeny.

The average pairwise SNP-distance between IHO-pig cluster isolates was 24 SNP's (95% CI: 19.33, 28.67), and the average pairwise SNP-distance between transmission cluster isolates was 24.6 SNP's (95% CI: 23.2, 25.9) and 10.8 SNP's (95% CI: 6.67, 14.93) (Table 1M.2). These results showed that transmission clusters displayed a high degree of phylogenetic relatedness between IHO pig and human LA-*S. aureus* CC9 isolates.

Growth curves for SF8300, NCHW8, IHW398-1, IHW398-2, and NCHW9 isolates.

No significant differences were observed between the optical densities of any *S. aureus* strain at any time point (Figure 2M.1). These growth curves established two (2) critical pieces of information: 1). CA-MRSA and LA-*S. aureus* do not differ in growth kinetics *in vivo*, and 2). The mid-logarithmic phase time point for each *S. aureus* strain

(2.5 hours). These results are summarized in the Chapter 2 main text, but the figure detailing growth curve results is provided only in the Miscellaneous Methods and Results section.

Candidate* S. aureus *antigens.

Fourteen (14) *S. aureus* candidate antigens were identified through literature search (Table 3M.1), composed of *S. aureus* proteins playing a role in colonization/adhesion, immune evasion, or infection/toxicity. Five (5) of these antigens were selected for further development into a multiplex *S. aureus* immunoassay for application in OF samples.

Coupling confirmation for* S. aureus *antigen xMap™ beads using pre- and post-infection mouse serum.

The 5-plex *S. aureus* immunoassay was used to probe pre-infection, acute-phase (14 days post-infection), and convalescent-phase (28 days post-infection) mouse sera from 5 different groups of mice, each infected with a different *S. aureus* strain. Serum IgG antibody levels against ClfA, ClfB, AT, and BT were, in general, elevated in acute-phase and convalescent-phase serum samples compared to pre-infection samples (Figure 3M.1). Serum IgG antibody levels against SCIN were very low (Figure 3M.1), likely due to the inactivity of SCIN in a mouse model. Elevated mouse serum IgG antibody levels against ClfA, ClfB, AT, and BT at 14 and 28 days post-*S. aureus* infection confirms that each bead set was successfully covalently coupled to its respective *S. aureus* antigen. These results are summarized in the Chapter 3 main text, but no figure is provided in Chapter 3.

Preliminary association between* S. aureus *nasal carriage status and oral fluid IgA and IgG antibody levels against SCIN, ClfA, ClfB, AT, and BT.

22 OF samples were probed for IgA and IgG antibody levels against SCIN, ClfA, ClfB, AT, and BT. IgA antibody levels against ClfA and BT were significantly elevated among persistent *S. aureus* nasal carriers compared to non-carriers ($p < .05$) (Table 3M.2). No antigen-specific IgG antibodies were significantly elevated among persistent *S. aureus* nasal carriers compared to non-carriers. These results informed the decision to include the ClfA coupled-bead set in the research outlined in Chapter 3.

Correlation between OF IgA and IgG antibody levels against SCIN, ClfA, and AT.

To determine whether OF IgA and IgG levels were correlated, we compared OF IgA and IgG antibody levels for each antigen specific target (SCIN, ClfA, and AT). In general, OF anti-SCIN, anti-ClfA, and anti-AT IgA and IgG levels were well correlated (anti-SCIN: $r = .4449$, $p = .000$; anti-ClfA: $r = .4279$, $p = .000$; *anti-AT:* $r = .4385$, $p = .000$) (Figure 3M.2). This dissertation does not expand further on the correlation between OF IgA and IgG antibody levels directed against *S. aureus* antigens. Future studies should consider the ratio of OF IgA to IgG antibody levels, and examine the relationship between age and the ratio of antigen-specific OF IgA and IgG antibody levels.

Table 1M.1. Quality metrics for whole genome sequencing datasets.

Length_cov_base = length of S. aureus genome at 0x; Length_cov_comp = length of S. aureus genome at 25x; genuses detected = names of genuses detected in sequenced library.

Sample name	num_of_reads	trimmed_num of_reads	read_length	species_ncbi	genuses_detected	un-classified	no of genuses	insert_size	insert_deviation	cov_base	cov comp	length_cov_base	length_cov_comp	length_differene	total_cov	N50	N75	Failed_filter count	called_snps
CRA1	882765	867663	301	Staphylococcus aureus	G Staphylococcus	2.74	1	257.57	896,396	0	25	'2765507	2765507	0	124.5	49661	30486	47	1
CRA2	1113048	1091955	301	Staphylococcus aureus	G Staphylococcus	1.81	1	274,988	952,606	0	25	2757757	2757757	0	163.7	52895	26265	21	0
IHOC1	511597	499756	301	Staphylococcus aureus	G Staphylococcus	2.04	1	311,231	109,059	0	25	2755063	2755063	0	78.6	31937	18756	30	5
IHOC2	578458	567002	301	Staphylococcus aureus	G Staphylococcus	1.67	1	316,727	111.58	0	25	2743445	2743445	0	91	73442	37203	43	1
IHOC3	484525	473260	301	Staphylococcus aureus	G Staphylococcus G	1.79	1	322,484	102,259	0	25	2771071	2771071	0	76.1	51623	30452	35	0
IHOP1	742410	712284	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	2.21	2	235,308	890,552	0	25	2742640	2742640	0	85.1	231352	127168	27	2
IHOP10	786381	744502	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	2.35	2	232,981	870,182	0	25	2749925	2749925	0	88.3	154222	70068	46	0
IHOP2	427722	377566	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	4.42	2	229,813	841,536	0	25	2745804	2710877	34927	42.5	107115	53464	38	1
IHOP3	326223	287841	301	Staphylococcus aureus	Staphylococcus,G Alteromonas	1.88	2	333,195	145,306	0	25	2740697	2737728	2969	43.3	154603	65480	46	0
IHOP4	396735	389055	301	Staphylococcus aureus	G Staphylococcus	2.17	1	346,538	119,191	0	25	2745162	2745162	0	63.2	55871	31448	32	0
IHOP5	203301	198832	301	Staphylococcus aureus	G Staphylococcus	1.96	1	321,635	103,759	0	25	2738907	2738907	0	33	203063	130114	29	0
IHOP6	527655	519279	301	Staphylococcus aureus	G Staphylococcus G	1.69	1	317,605	107,689	0	25	2739847	2739847	0	83.8	68329	34324	36	0
IHOP7	535879	507443	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	4.1	2	235,098	891,131	0	25	2817867	2817867	0	59.2	150025	80023	47	0
IHOP8	579367	530183	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	5.51	2	233,557	869,022	0	25	2768557	2768557	0	62.8	224462	104813	41	0
IHOP9	597662	576900	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	5.86	2	234,232	874,311	0	25	2811983	2811983	0	67.2	138166	80064	51	2
IHOW1	274013	249175	301	Staphylococcus aureus	Staphylococcus,G Alteromonas G	2.28	2	346,448	155.35	0	25	2743441	2729429	14012	37.1	319158	130053	36	4
IHOW10	351859	229377	301	Staphylococcus aureus	Staphylococcus,G Alteromonas	5.56	2	342,964	154,908	0	25	2750823	2750823	0	37.3	331706	127073	23	0
IHOW11	441596	431371	301	Staphylococcus aureus	G Staphylococcus G	3.32	1	321,851	107,149	0	25	2802081	2802081	0	68.3	49733	26080	32	2
IHOW12	236702	216454	301	Staphylococcus aureus	Staphylococcus,G Alteromonas	1.58	2	337,982	145,396	0	25	2696642	2696642	0	33.5	280243	80855	48	2
IHOW13	639189	629189	301	Staphylococcus aureus	G Staphylococcus	1.9	1	323.55	107,478	0	25	2698398	2698398	0	103.6	70350	52429	37	1

IHOW14	467277	457796	301	Staphylococcus aureus	G Staphylococcus G	6.38	1	260,868	954,888	0	25	2746882	2737637	9245	64.2	151175	56167	49	0
IHOW15	269230	240487	301	Staphylococcus aureus	Staphylococcus,G Alteromonas	1.76	2	339,341	150,972	0	25	2743605	2743605	0	37	379027	176212	20	0
IHOW16	429757	419279	301	Staphylococcus aureus	G Staphylococcus	2.95	1	326,729	107,317	0	25	2706984	2706984	0	68.2	34719	16641	30	0
IHOW17	478127	468565	301	Staphylococcus aureus	G Staphylococcus G	2.2	1	330.86	110.72	0	25	2749045	2749045	0	76.6	73522	37203	35	2
IHOW18	345748	286939	301	Staphylococcus aureus	Staphylococcus,G Alteromonas G	2.16	2	331.09	135,468	0	25	2754547	2731782	22765	47.8	115689	53504	20	0
IHOW19	514782	462699	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	3.79	2	255,278	101,476	0	25	2743438	2743438	0	58.5	150675	68337	42	0
IHOW2	259968	226852	301	Staphylococcus aureus	Staphylococcus,G Alteromonas	5.35	2	340,071	153,226	0	25	2768658	1778155	990503	34.1	152191	73314	40	0
IHOW20	273655	267164	301	Staphylococcus aureus	G Staphylococcus G	3.91	1	344,781	109,756	0	25	2788636	2788636	0	43.7	280149	114104	30	0
IHOW21	298736	196387	301	Staphylococcus aureus	Staphylococcus,G Alteromonas G	6.78	2	385,355	163,192	0	25	2750408	2750408	0	35.1	280243	124596	23	1
IHOW22	573604	416161	301	Staphylococcus aureus	Staphylococcus,G Alteromonas G	8.75	2	358.74	149.93	0	25	2785520	2785520	0	70.4	62372	34599	49	0
IHOW23	444450	377740	301	Staphylococcus aureus	Staphylococcus,G Alteromonas	4.54	2	376,764	157,352	0	25	2713392	2713392	0	68.2	280185	151266	39	3
IHOW24	416596	407283	301	Staphylococcus aureus	G Staphylococcus G	4.25	1	343,042	111,839	0	25	2780690	2780690	0	66.3	76196	40769	46	0
IHOW25	409385	354606	300	Staphylococcus aureus	Staphylococcus,G Alteromonas	3.99	2	261,923	107,755	0	25	2732208	2732208	0	44.8	162447	76810	50	0
IHOW26	370219	362010	301	Staphylococcus aureus	G Staphylococcus G	1.72	1	334,051	104,977	0	25	2744379	2744379	0	59.2	43386	26001	27	3
IHOW27	274901	197258	301	Staphylococcus aureus	Staphylococcus,G Alteromonas G	6.91	2	354,423	148,595	0	25	2713265	2663882	49383	34.2	305473	126029	29	0
IHOW28	328932	288558	301	Staphylococcus aureus	Staphylococcus,G Alteromonas G	10.04	2	350,546	159,731	0	25	2768059	2463604	304455	47.2	174526	85030	34	1
IHOW29	543465	482537	300	Staphylococcus aureus	Staphylococcus,G Alteromonas G	3.68	2	247,019	973,943	0	25	2730801	2730801	0	59.6	151223	63621	49	0
IHOW3	249579	191893	301	Staphylococcus aureus	Staphylococcus,G Alteromonas	3.88	2	389,892	166,303	0	25	2751311	2701928	49383	34	283087	114580	19	0
IHOW30	525369	514581	301	Staphylococcus aureus	G Staphylococcus	1.74	1	316,105	109,865	0	25	2742687	2742687	0	81.9	61854	36052	35	0
IHOW31	970905	896350	102	Staphylococcus aureus	G Staphylococcus	2.42	1	430,573	634,584	0	25	2706863	2705294	1569	64.5	220083	73175	54	41
IHOW32	3038210	2812586	102	Staphylococcus aureus	G Staphylococcus	3.63	1	423.15	685,722	0	25	2735008	2715140	19868	194.3	140824	61700	78	41
IHOW33	348971	338842	251	Staphylococcus aureus	G Staphylococcus	2.91	1	300,608	728,277	0	25	2822362	2822362	0	55.1	297424	114117	28	0

IHOW34	551014	541521	301	Staphylococcus aureus	G Staphylococcus	1.65	1	269,602	100.51	0	25	2752608	2752608	0	80.2	65342	38056	50	0
IHOW4	644311	635408	301	Staphylococcus aureus	G Staphylococcus	1.64	1	293.43	946,233	0	25	2748653	2748653	0	99.9	50636	31071	31	2
IHOW5	360246	340228	301	Staphylococcus aureus	G Staphylococcus	5.73	1	349,383	136.19	0	25	2796134	2796134	0	56.6	20883	12645	43	5
IHOW6	748398	715835	301	Staphylococcus aureus	G Staphylococcus	1.33	1	375,213	151,892	0	25	2758820	2758820	0	126.4	36934	20192	35	5
IHOW7	547047	536337	301	Staphylococcus aureus	G Staphylococcus	1.7	1	275,267	930,832	0	25	2751044	2746545	4499	80.2	36235	21062	35	0
IHOW8	316981	301339	301	Staphylococcus aureus	G Staphylococcus	2.62	1	368,033	165,973	0	25	2723580	2722285	1295	51	36377	19960	22	0
IHOW9	557707	545857	301	Staphylococcus aureus	G Staphylococcus	1.69	1	297,413	104,981	0	25	2702388	2702388	0	86.4	73466	34707	36	0

Cluster	No. of isolates	Range of pairwise SNP-distances	Average pairwise SNP-distance	95% Confidence interval
IHO pig cluster	6	2-42	24	19.3-28.7
Transmission cluster A	14	0-42	24.6	23.2-25.9
Transmission cluster B	5	1-25	10.8	6.67-14.9

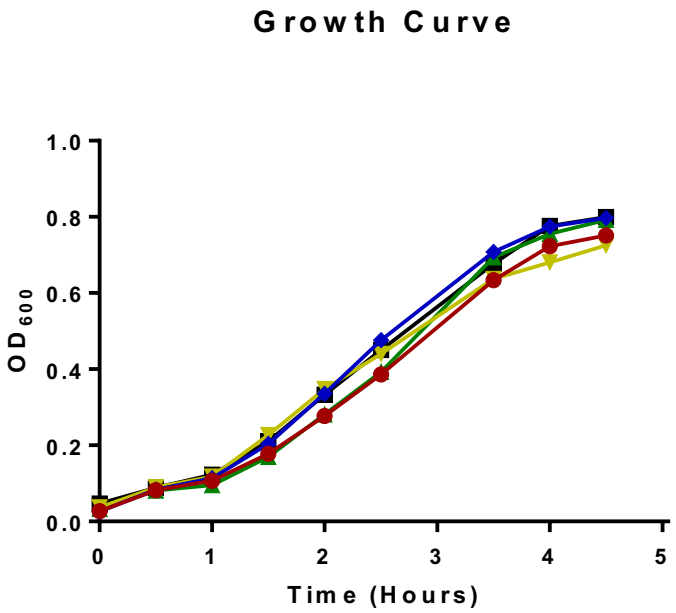
Table 1M.2. Table of average pairwise SNP-distances by cluster in high-resolution phylogeny. The range of pairwise SNP-distances calculated for the IHO pig cluster was used to set a threshold for transmission and identify transmission clusters with intermingled IHO pig and human LA-*S. aureus* CC9 isolates separated by 42 SNP's or less.

***S. aureus* antigens of known immunogenicity and role in pathogenesis**	
Stage	**Antigen/Antibody**
Colonization	Clumping factor A (ClfA)[99]
	Clumping factor B (ClfB)[99]
	Fibronectin binding protein A (FnbpA)[123]
	Fibronectin binding protein B (FnbpB)[123]
Host defense evasion	Staphylococcal complement inhibitor (SCIN)[99]
	Formyl peptide receptor-like inhibitory protein (FLIPr-L)[123]
	Staphylococcal superantigen-like protein 5 (SSL5)[123]
Infection/toxicity	Alpha hemolysin (Hla)[27]
	Beta hemolysin (Hlb)
	Panton-Valentine Leukocidin (PVL)[124]
	Staphylococcal enterotoxin A[99,123]
	Staphylococcal enterotoxin B[99,123]
	Toxic shock syndrome toxin-1[99]

Table 3M.1. Table of candidate *S. aureus* antigens with references. Listed antigens are supported in prior literature as biomarkers of *S. aureus* colonization or infection. References are provided for each listed *S. aureus* protein.

		Carrier Mean (CI) n=11	**Non-carrier** Mean (CI) n=11	***p*-value**
IgG				
	anti-ClfA	3175.84 (494.78, 5856.89)	2994.66 (1083.22, 4906.09)	0.9036
	anti-ClfB	1664.34 (558.51, 2770.17)	2583.61 (1074.89, 4092.32)	0.2865
	anti-AT	11652.36 (6520.76, 16783.96)	11219.23 (6304.31, 16134.15)	0.8933
	anti-BT	13588.47 (6940.95, 20236)	12526.38 (6855.37, 18197.39)	0.7893
	anti-SCIN	6846.59 (2336.845, 11356.34)	6690.23 (2442.91, 10937.54)	0.9557
IgA				
	anti-**ClfA**	1002.773 (197.58, 1807.96)	167.14 (68.87, 265.40)	**0.0327**
	anti-ClfB	179.15 (38.73, 319.58)	117.8364 (30.73, 204.95)	0.4181
	anti-AT	572.47 (-30.08, 1175.028)	316.47 (69.99, 562.95)	0.3913
	anti-**BT**	1875. 16 (791.79, 2958.53)	753.85 (251.85, 1255.837)	**0.0494**
	anti-SCIN	1469.38 (365.06, 2573.70)	1146.34 (425.39, 1867.28)	0.5913

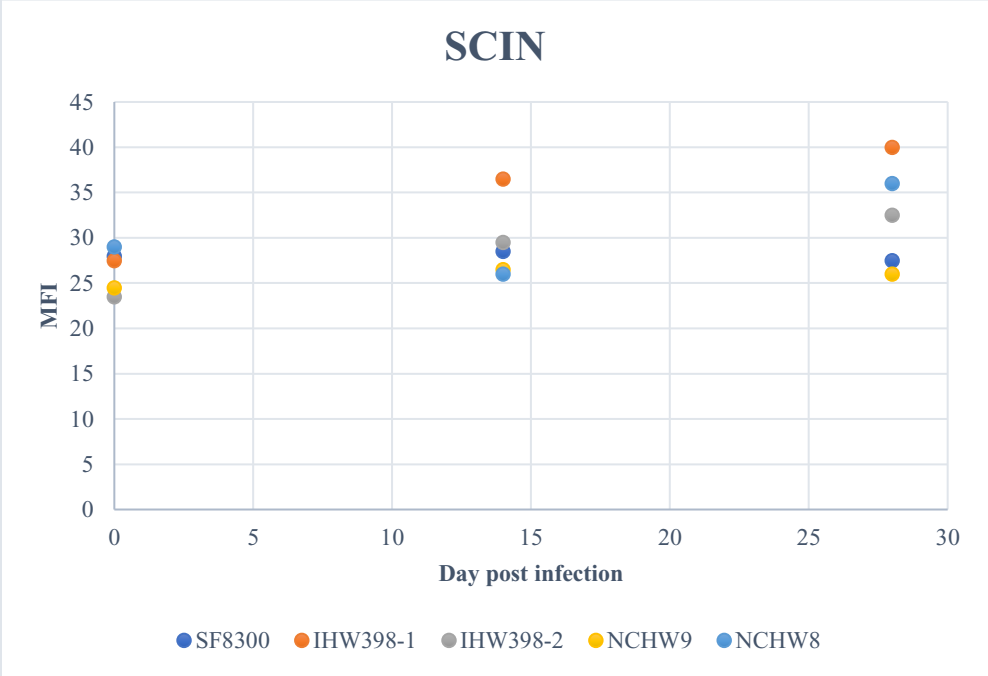
Table 3M.2. Preliminary association between *S. aureus* nasal carriage status and oral fluid IgA and IgG antibody levels against SCIN, ClfA, ClfB, AT, and BT. Geometric means and 95% confidence intervals are provided for oral fluid IgA and IgG antibody levels against ClfA, ClfB, AT, BT, and SCIN. *p* values were estimated using a students *t* test, comparing the average antibody levels among carriers to that of non-carriers, for each *S. aureus* antigen target. $p < .05$ = statistically significant, and are bolded.



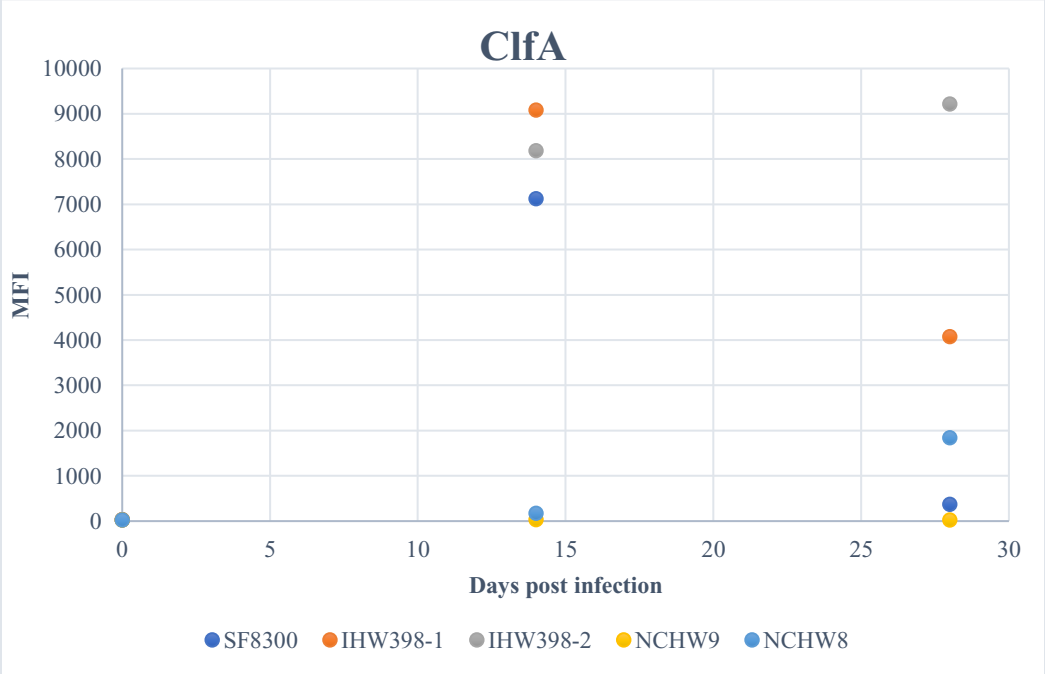
<u>Figure 2M.1.</u> Growth curves for SF8300, NCHW8, IHW398-1, IHW398-2, and NCHW9 *S. aureus* isolates. No statistically significant differences were observed between OD600 of any *S. aureus* strain at any time point. Mid-logarithmic phase for all *S. aureus* strains determined to be 2.5 hours. *Note.* Black = SF8300; red = IHW398-1; blue = IHW398-2; green = NCHW9; yellow = NCHW8.

<u>Figure 3M.1.</u> Coupling confirmation for *S. aureus* antigen xMap™ beads using pre- and post- infection mouse serum. Absolute MFI values are provided for each serum time point, for each *S. aureus* antigen target. Serum IgG levels against ClfA, AT, and BT were especially strong at 14 and 28 days post-infection. BT was not included in the *S. aureus* immunoassay described in Chapter 3 because it can be disrupted by prophage integration in humans[55]. **A).** Mouse serum anti-SCIN antibody levels; **B).** Mouse serum anti-ClfA antibody levels; **C).** Mouse serum anti-ClfB antibody levels; **D).** Mouse serum anti-AT antibody levels; **E).** Mouse serum anti-BT antibody levels. All bead sets were run in multiplex (5-plex). *Note.* SCIN = staphylococcal complement inhibitor; ClfA = clumping factor A; ClfB = clumping factor B; AT = alpha toxin; BT = beta toxin; Day 0 = pre-infection; Day 14 = acute-phase infection; Day 28 = convalescent-phase infection. SF8300 = serum from SF8300 infected mouse; IHW398-1 = serum from IHW398-1 infected mouse; IHW398-2 = serum from IHW398-2 infected mouse; NCHW9 = serum from NCHW9 infected mouse; NCHW8 = serum from NCHW8 infected mouse.

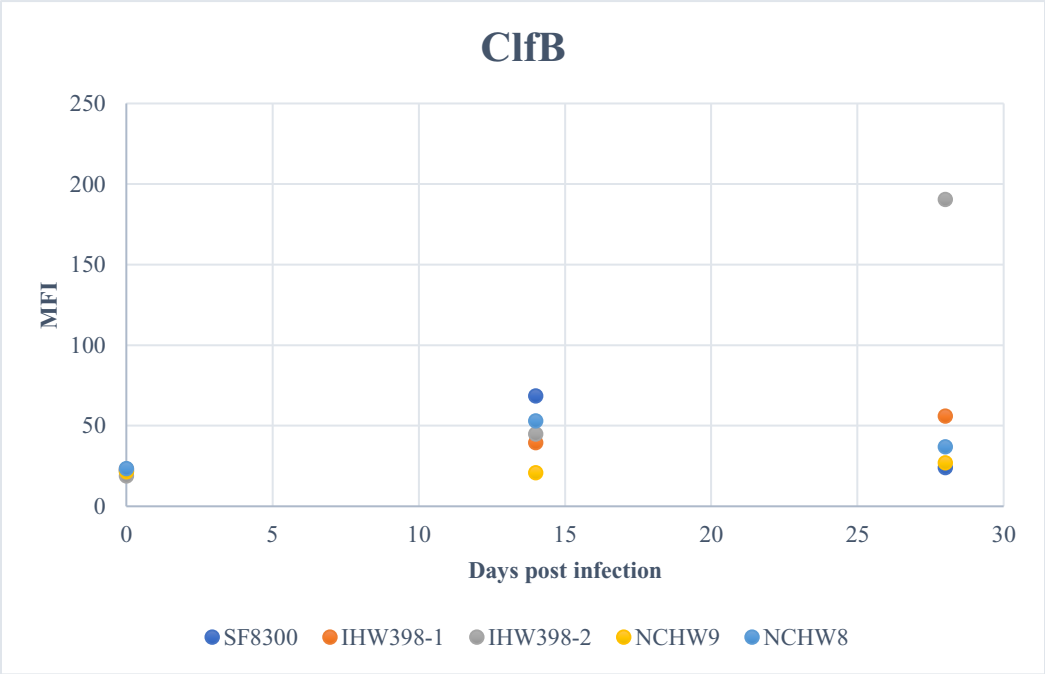
A).


SCIN
MFI
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Day post infection
SF8300
IHW398-1
IHW398-2
NCHW9
NCHW8

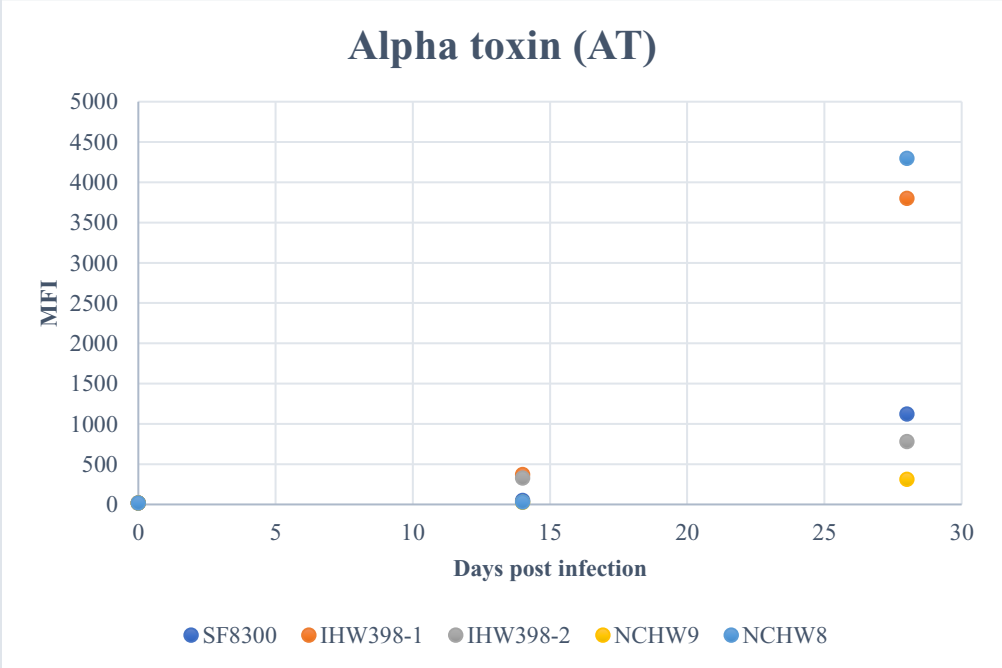
B).


ClfA
MFI
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
Days post infection
SF8300
IHW398-1
IHW398-2
NCHW9
NCHW8

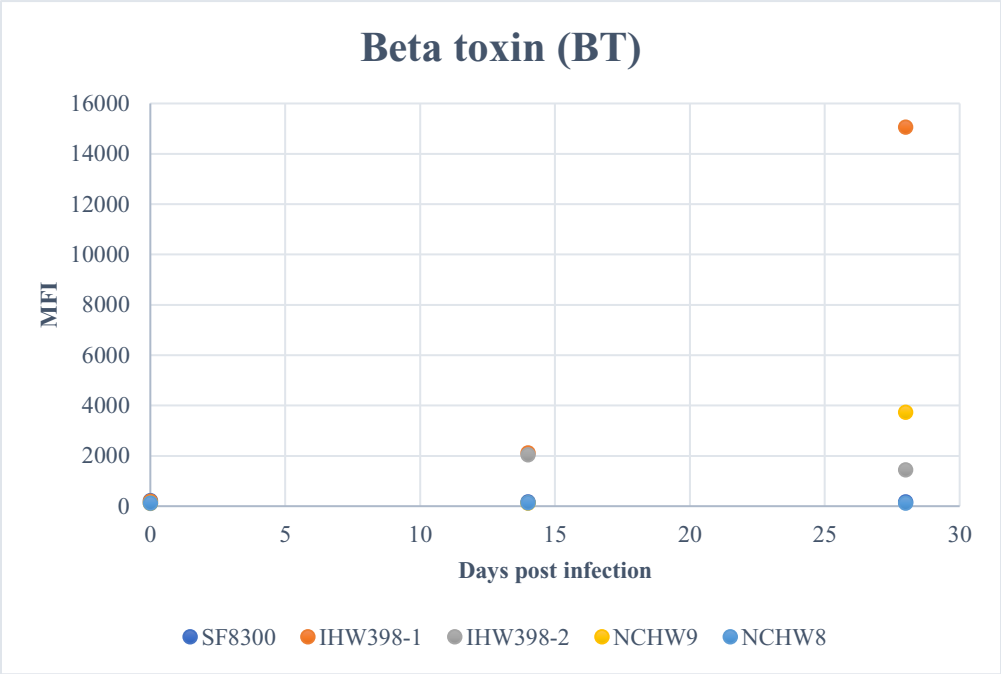
C).


ClfB
250
200
150
100
50
0
MFI
0
5
10
15
20
25
30
Days post infection
SF8300
IHW398-1
IHW398-2
NCHW9
NCHW8

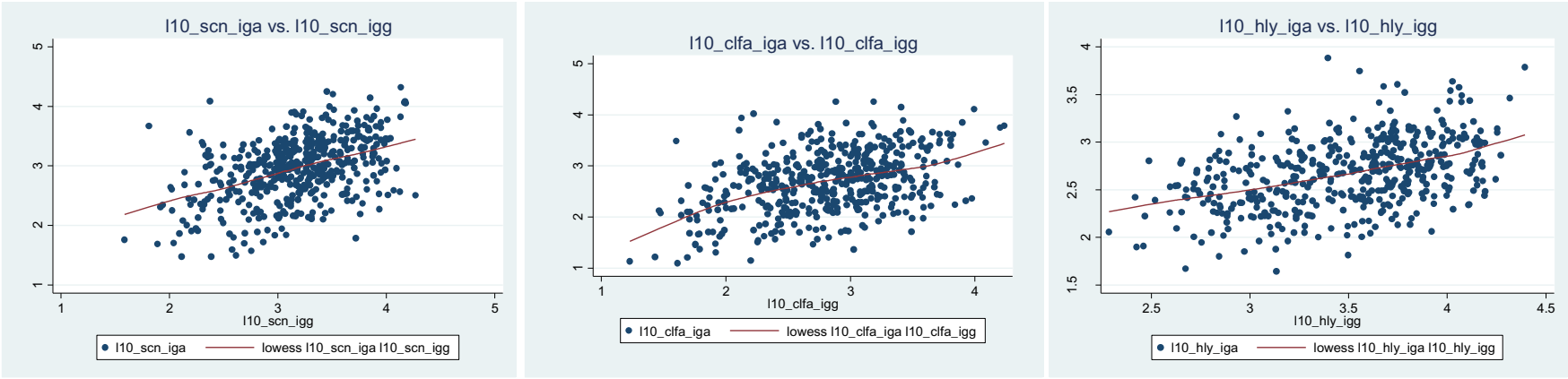
D).


Alpha toxin (AT)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
MFI
0
5
10
15
20
25
30
Days post infection
SF8300
IHW398-1
IHW398-2
NCHW9
NCHW8

E).


Beta toxin (BT)
16000
14000
12000
10000
8000
6000
4000
2000
0
MFI
0
5
10
15
20
25
30
Days post infection
SF8300
IHW398-1
IHW398-2
NCHW9
NCHW8

A). B). C).



<u>Figure 3M.2.</u> Correlation between OF IgA and IgG antibody levels against SCIN, ClfA, and AT with LOWESS curve. A). Correlation between OF anti-SCIN IgG and anti-SCIN IgA. B). Correlation between OF anti-ClfA IgG and anti-ClfA IgA. C). Correlation between OF anti-AT IgG and anti-AT IgA.

REFERENCES

1. Mcallister-hollod, L., Nadle, J., Ray, S. M., Thompson, D. L. & Wilson, L. E. Multistate Point-Prevalence Survey of Health Care–Associated Infections. 1198–1208 (2019). doi:10.1056/NEJMoa1306801

2. Gorwitz, R. J. *et al.* Changes in the Prevalence of Nasal Colonization with Staphylococcus aureus in the United States , 2001 – 2004. **197,** 2001–2004 (2008).

3. Deleo, F. R., Otto, M., Kreiswirth, B. N. & Chambers, H. F. Staphylococcus aureus. *Lancet* **375,** 1557–1568 (2010).

4. Daum, R. S. Skin and Soft-Tissue Infections Caused by Methicillin-Resistant Staphylococcus aureus. (2019).

5. Dantes, R. *et al.* National Burden of Invasive Methicillin-Resistant. **30333,** 1970–1978 (2013).

6. Areas, H., Population, H. & Definition, H. C. Healthcare-Associated Infections-Community Interface (HAIC): Emerging Infections Program (EIP) Network Report Invasive Staphylococcus aureus , 2016. 2009–2016 (2018).

7. Herold, B. C. *et al.* Staphylococcus aureus in Children With No Identified Predisposing Risk. **279,** 593–598 (1998).

8. Infections, M. S., Mcdougal, L. K., Carey, R. B., Ph, D. & Talan, D. A. S. aureus. 666–674 (2019).

9. Rinsky, J. L. *et al.* Livestock-Associated Methicillin and Multidrug Resistant Staphylococcus aureus Is Present among Industrial, Not Antibiotic-Free Livestock Operation Workers in North Carolina. *PLoS One* **8,** 1–11 (2013).

10. Hatcher, S. M. *et al.* The prevalence of antibiotic-resistant Staphylococcus aureus nasal carriage among industrial hog operation workers, community residents, and children living in their households: North carolina, USA. *Environ. Health Perspect.* **125,** 560–569 (2017).

11. Casey, J. A., Curriero, F. C., Cosgrove, S. E., Nachman, K. E. & Schwartz, B. S. High-density livestock operations, crop field application of manure, and risk of community-associated

methicillin-resistant Staphylococcus aureus infection in Pennsylvania. *JAMA Intern. Med.* **173,** 1980–1990 (2013).

12. Price, L. B. *et al.* Adaptation and emergence of Staphylococcus aureus CC39: Host adaptation and emergence of methicillin resistance in livestock. *MBio* **3,** 1–6 (2012).

13. van Cleef, B. A. G. L. *et al.* Livestockassociated methicillin- resistant staphylococcus aureus in humans, Europe. *Emerg. Infect. Dis.* **17,** 502–505 (2011).

14. Nadimpalli, M. L. *et al.* Face Mask Use and Persistence of Livestock-associated *Staphylococcus aureus* Nasal Carriage among Industrial Hog Operation Workers and Household Contacts, USA. *Environ. Health Perspect.* **126,** 127005 (2018).

15. Nadimpalli, M. *et al.* Livestock-associated, antibiotic-resistant Staphylococcus aureus nasal carriage and recent skin and soft tissue infection among industrial HOG operation workers. *PLoS One* **11,** 1–16 (2016).

16. Wardyn, S. E. *et al.* Swine farming is a risk factor for infection with and high prevalence of carriage of multidrug-resistant staphylococcus aureus. *Clin. Infect. Dis.* **61,** 59–66 (2015).

17. Davis, M. F. *et al.* Occurrence of Staphylococcus aureus in swine and swine workplace environments on industrial and antibiotic-free hog operations in North Carolina, USA: A One Health pilot study. *Environ. Res.* **163,** 88–96 (2018).

18. Gibbs, S. G. *et al.* Isolation of Antibiotic-Resistant Bacteria from the Air Plume Downwind of a Swine Confined or Concentrated Animal Feeding Operation. **1032,** 1032–1037 (2006).

19. Uhlemann, A.-C. *et al.* Identification of a Highly Transmissible Animal-Independent. *MBio* **3,** 1–9 (2012).

20. Shepheard, M. A. *et al.* Historical Zoonoses and Other Changes in Host Tropism of Staphylococcus aureus, Identified by Phylogenetic Analysis of a Population Dataset. *PLoS One* **8,** 1–9 (2013).

21. Wassenberg, M. W. M., Bootsma, M. C. J., Troelstra, A., Kluytmans, J. A. J. W. & Bonten, M. J. M. Transmissibility of livestock-associated methicillin-resistant Staphylococcus aureus (ST398) in Dutch hospitals. *Clin. Microbiol. Infect.* **17,** 316–319 (2011).

22. Bootsma, M. C. J., Wassenberg, M. W. M., Trapman, P. & Bonten, M. J. M. The nosocomial transmission rate of resistant Staphylococcus aureus. 1–7 (2010).

23. Smith, T. C. Livestock-Associated Staphylococcus aureus: The United States Experience. *PLoS Pathog.* **11,** 1–8 (2015).

24. Golding, G. R. *et al.* Livestock-associated methicillin-resistant Staphylococcus aureus sequence type 398 in humans, Canada. *Emerg. Infect. Dis.* **16,** 587–594 (2010).

25. Larsen, J. *et al.* Emergence of Livestock-Associated Methicillin-Resistant Staphylococcus aureus Bloodstream Infections in Denmark. *Clin. Infect. Dis.* **65,** 1072–1076 (2017).

26. van Belkum, A. Methicillin-Resistant and -Susceptible Staphylococcus aureus Sequence Type 398 in Pigs and Humans. *Emerg. Infect. Dis.* **14,** 479–483 (2008).

27. Wu, Y. *et al.* Prevalence of IgG and neutralizing antibodies against Staphylococcus aureus alphatoxin in healthy human subjects and diverse patient populations. *Infect. Immun.* **86,** 1–10 (2018).

28. Butaye, P., Argudín, M. A. & Smith, T. C. Livestock-Associated MRSA and Its Current Evolution. 19–31 (2016). doi:10.1007/s40588-016-0031-9

29. Chuang, Y. & Huang, Y. International Journal of Antimicrobial Agents Livestock-associated meticillin-resistant Staphylococcus aureus in Asia : An emerging issue ? *Int. J. Antimicrob. Agents* **45,** 334–340 (2015).

30. Ye, X. *et al.* Genotypic and Phenotypic Markers of Livestock-Associated Methicillin-Resistant Staphylococcus aureus CC9 in Humans. **82,** 3892–3899 (2016).

31. Larsen, J. *et al.* Meticillin-resistant Staphylococcus aureus CC398 is an increasing cause of disease in people with no livestock contact in Denmark , 1999 to 2011. (2015).

32. Collie. Research letters. *Br J Sport. Med* **24,** 370–376 (2003).

33. Data, C. North Carolina. **1,** (2014).

34. Boucher, H. W. & Corey, G. R. Epidemiology of Methicillin-Resistant Staphylococcus aureus INFECTIONS. **02111,** (2008).

35. Klevens, R. M. *et al.* Invasive Methicillin-Resistant Staphylococcus aureus Infections in the

United States. **298,** 1763–1771 (2007).

36. Miller, L. S. & Cho, J. S. Immunity against Staphylococcus aureus cutaneous infections. *Nat. Publ. Gr.* **11,** 505–518 (2011).

37. Diep, B. A. *et al.* Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. *Lancet* **367,** 731–739 (2006).

38. Li, M. *et al.* Evolution of virulence in epidemic community-associated methicillin-resistant Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 5883–8 (2009).

39. Iqbal, Z. *et al.* Comparative virulence studies and transcriptome analysis of Staphylococcus aureus strains isolated from animals. *Sci. Rep.* **6,** 1–12 (2016).

40. Holtfreter, S., Kolata, J. & Br, B. M. International Journal of Medical Microbiology Towards the immune proteome of Staphylococcus aureus – The anti- S . aureus antibody response. **300,** 176–192 (2010).

41. Adhikari, R. P. *et al.* Lower antibody levels to staphylococcus aureus exotoxins are associated with sepsis in hospitalized adults with invasive S. aureus infections. *J. Infect. Dis.* **206,** 915–923 (2012).

42. Ruotsalainen, E. *et al.* Methicillin-sensitive Staphylococcus aureus bacteraemia and endocarditis among injection drug users and nonaddicts: Host factors, microbiological and serological characteristics. *J. Infect.* **56,** 249–256 (2008).

43. Fritz, S. A. *et al.* A serologic correlate of protective immunity against community-onset staphylococcus aureus infection. *Clin. Infect. Dis.* **56,** 1554–1561 (2013).

44. Smith, D. L., Dushoff, J. & Morris, J. G. Agricultural Antibiotics and Human Health Does antibiotic use in agriculture have a greater impact than hospital use ? **2,** (2005).

45. Smith, T. C. *et al.* Methicillin-Resistant Staphylococcus aureus (MRSA) Strain ST398 Is Present in Midwestern U . S . Swine and Swine Workers. **4,** 1–6 (2009).

46. Van Loo, I. *et al.* Emergence of methicillin-resistant Staphylococcus aureus of animal origin in humans. *Emerg. Infect. Dis.* **13,** 1834–1839 (2007).

47. Nadimpalli, M., Heaney, C. & Stewart, J. R. Identification of Staphylococcus aureus from enriched nasal swabs within 24 h is improved with use of multiple culture media. *J. Med. Microbiol.* **62,** 1365–1367 (2013).

48. Sahl, J. W. *et al.* Methods Paper NASP : an accurate , rapid method for the identification of SNPs in WGS datasets that supports flexible input and output formats. 1–12 (2019). doi:10.1099/mgen.0.000074

49. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows – Wheeler transform. **25,** 1754–1760 (2009).

50. Mckenna, A. *et al.* The Genome Analysis Toolkit : A MapReduce framework for analyzing next-generation DNA sequencing data. 1–8 (2010). doi:10.1101/gr.107524.110.20

51. Croucher, N. J. *et al.* Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. **43,** (2015).

52. Uindon, P. G. & Ranc, J. E. A. N. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies : Assessing the Performance of PhyML 3 . 0. **59,** 307–321 (2010).

53. Joensen, K. G. *et al.* Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. *J. Clin. Microbiol.* **52,** 1501–1510 (2014).

54. Zankari, E. *et al.* Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* **67,** 2640–2644 (2012).

55. Wamel, W. J. B. Van, Rooijakkers, S. H. M., Kessel, K. P. M. Van, Strijp, J. a G. Van & Ruyken, M. The Innate Immune Modulators Staphylococcal Complement Inhibitor and Chemotaxis Inhibitory Protein of Staphylococcus aureus Are Located on β The Innate Immune Modulators Staphylococcal Complement Inhibitor and Chemotaxis Inhibitory Protein of Staphylococc. **188,** 1310–1315 (2006).

56. Zhou, W. *et al.* WGS analysis of ST9-MRSA-XII isolates from live pigs in China provides insights into transmission among porcine, human and bovine hosts. 2652–2661 (2018). doi:10.1093/jac/dky245

57. Fluit, A. C., Wielders, C. L. C., Verhoef, J. & Homer, H. Epidemiology and Susceptibility of 3

, 051 Staphylococcus aureus Isolates from 25 University Hospitals Participating in the European SENTRY Study. **39,** 3727–3732 (2001).

58. Larsen, J. *et al.* Copresence of tet (K) and tet (M) in Livestock-Associated Methicillin-Resistant Staphylococcus aureus Clonal Complex 398 Is Associated with Increased Fitness during Exposure to Sublethal Concentrations of. **60,** 4401–4403 (2016).

59. Trzcinski, K., Cooper, B. S., Hryniewicz, W. & Dowson, C. G. JAC Original articles Expression of resistance to tetracyclines in strains of methicillin-resistant Staphylococcus aureus. 763–770 (2000).

60. Haroche, J., Allignet, J., Buchrieser, C. & Pathoge, M. Characterization of a Variant of vga (A) Conferring Resistance to Streptogramin A and Related Compounds. **44,** 2271–2275 (2000).

61. Gentry, D. R. *et al.* Genetic Characterization of Vga ABC Proteins Conferring Reduced Susceptibility to Pleuromutilins in Staphylococcus aureus □. **52,** 4507–4509 (2008).

62. Mendes, R. E. *et al.* Plasmid-borne vga (A) -encoding gene in methicillin-resistant Staphylococcus aureus ST398 recovered from swine and a swine farmer in the United States ☆ , ☆☆ , ★. *Diagn. Microbiol. Infect. Dis.* **71,** 177–180 (2011).

63. Cole, D., Todd, L. & Wing, S. Reviews2 e. **1081,** (2000).

64. Stimson, J. *et al.* Beyond the SNP threshold : identifying outbreak clusters using inferred transmissions. 1–29 (2018).

65. Larsen, J., Stegger, M., Andersen, P. S. & Petersen, A. Evidence for Human Adaptation and Foodborne Transmission of Livestock- Associated Methicillin-Resistant Staphylococcus aureus. **63,** (2016).

66. Strauß, L. *et al.* Origin , evolution , and global transmission of community-acquired Staphylococcus aureus ST8. (2017). doi:10.1073/pnas.1702472114

67. Durand, G. *et al.* Routine Whole-Genome Sequencing for Outbreak Investigations of Staphylococcus aureus in a National Reference Center. **9,** 1–12 (2018).

68. Tong, S. Y. C. *et al.* Genome sequencing defines phylogeny and spread of methicillin-resistant Staphylococcus aureus in a high transmission setting. 111–118 (2015).

doi:10.1101/gr.174730.114.Freely

69. Coll, F. *et al.* Longitudinal genomic surveillance of MRSA in the UK reveals transmission patterns in hospitals and the community. **9745,** (2017).

70. Schulz, J. *et al.* Longitudinal Study of the Contamination of Air and of Soil Surfaces in the Vicinity of Pig Barns by Livestock-Associated Methicillin- Resistant Staphylococcus aureus. **78,** 5666–5671 (2012).

71. Thapaliya, D., Kadariya, A., Lohani, S. & Smith, T. C. Prevalence and Molecular Characterization of S. aureus and MRSA on Children's Playgrounds. (2018). doi:10.1097/INF.0000000000002095

72. WHO. *Critically Important Antimicrobials for Human Medicine*. *World Health Organization* (2017). doi:10.1017/CBO9781107415324.004

73. Feingold, B. J. *et al.* Livestock Density as Risk Factor for Resistant Staphylococcus aureus , the Netherlands. *Emerg. Infect. Dis.* **18,** 1841–1849 (2012).

74. Argudín, M. A. *et al.* Virulence and resistance determinants of German Staphylococcus aureus ST398 isolates from nonhuman sources. *Appl. Environ. Microbiol.* **77,** 3052–3060 (2011).

75. Ballhausen, B. *et al.* LA-MRSA CC398 differ from classical community acquired-MRSA and hospital acquired-MRSA lineages: Functional analysis of infection and colonization processes. *Int. J. Med. Microbiol.* **304,** 777–786 (2014).

76. Rasigade, J. *et al.* Global Distribution and Evolution of Panton-Valentine Leukocidin–Positive Methicillin-Susceptible *Staphylococcus aureus,* 1981–2007. *J. Infect. Dis.* **201,** 1589–1597 (2010).

77. Valentin-Domelier, A. S. *et al.* Methicillin-susceptible ST398 Staphylococcus aureus responsible for bloodstream infections: An emerging human-adapted subclone? *PLoS One* **6,** (2011).

78. Mediavilla, J. R. *et al.* Methicillin-susceptible Staphylococcus aureus ST398, New York and New Jersey, USA. *Emerg. Infect. Dis.* **18,** 700–702 (2012).

79. Orscheln, R. C. *et al.* NIH Public Access. *October* **49,** 536–542 (2010).

80. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* **30,** 2114–2120 (2014).

81. Nurk, S. *et al.* Assembling Single-Cell Genomes and Mini-Metagenomes From Chimeric MDA Products. *J. Comput. Biol.* **20,** 714–737 (2013).

82. Gurevich, A., Saveliev, V., Vyahhi, N. & Tesler, G. QUAST: Quality assessment tool for genome assemblies. *Bioinformatics* **29,** 1072–1075 (2013).

83. Camacho, C. *et al.* BLAST+: Architecture and applications. *BMC Bioinformatics* **10,** 1–9 (2009).

84. Maiden, M. C. J. *et al.* Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci.* **95,** 3140–3145 (1998).

85. Foster, T. J., Geoghegan, J. A., Ganesh, V. K. & Höök, M. Adhesion, invasion and evasion: The many functions of the surface proteins of Staphylococcus aureus. *Nat. Rev. Microbiol.* **12,** 49–62 (2014).

86. Paharik, A. E. *et al.* The Spl Serine Proteases Modulate Staphylococcus aureus Protein Production and Virulence in a Rabbit Model of Pneumonia. *mSphere* **1,** 1–16 (2016).

87. Lewis, A. M. *et al.* Examination of the Staphylococcus aureus nitric oxide reductase (saNOR) reveals its contribution to modulating intracellular NO levels and cellular respiration. *Mol. Microbiol.* **96,** 651–669 (2015).

88. Vandenesch, F., Lina, G. & Henry, T. Staphylococcus aureus Hemolysins, bi-component Leukocidins, and Cytolytic Peptides: A Redundant Arsenal of Membrane-Damaging Virulence Factors? *Front. Cell. Infect. Microbiol.* **2,** 1–15 (2012).

89. Tkaczyk, C. *et al.* Staphylococcus aureus Alpha Toxin Suppresses Effective Innate and Adaptive Immune Responses in a Murine Dermonecrosis Model. *PLoS One* **8,** 1–12 (2013).

90. Sampedro, G. R. *et al.* Targeting Staphylococcus aureus α-toxin as a novel approach to reduce severity of recurrent skin and soft-tissue infections. *J. Infect. Dis.* **210,** 1012–1018 (2014).

91. Kobayashi, S. D. *et al.* Comparative analysis of USA300 virulence determinants in a rabbit model of skin and soft tissue infection. *J. Infect. Dis.* **204,** 937–941 (2011).

92. Spaan, A. N., Van Strijp, J. A. G. & Torres, V. J. Leukocidins: Staphylococcal bi-component pore-forming toxins find their receptors. *Nat. Rev. Microbiol.* **15,** 435–447 (2017).

93. Alonzo, F. & Torres, V. J. The Bicomponent Pore-Forming Leucocidins of Staphylococcus aureus. *Microbiol. Mol. Biol. Rev.* **78,** 199–230 (2014).

94. Cho, J. S. *et al.* Neutrophil-derived IL-1β Is Sufficient for Abscess Formation in Immunity against Staphylococcus aureus in Mice. *PLoS Pathog.* **8,** (2012).

95. Miller, L. S. *et al.* Inflammasome-Mediated Production of IL-1 Is Required for Neutrophil Recruitment against Staphylococcus aureus In Vivo. *J. Immunol.* **179,** 6933–6942 (2007).

96. Kobayashi, S. D., Malachowa, N. & Deleo, F. R. Pathogenesis of Staphylococcus aureus abscesses. *Am. J. Pathol.* **185,** 1518–1527 (2015).

97. Brady, R. A., Bruno, V. M. & Burns, D. L. RNA-seq analysis of the host response to Staphylococcus aureus skin and soft tissue infection in a mouse model. *PLoS One* **10,** 1–31 (2015).

98. Katayama, Y., Baba, T., Sekine, M., Fukuda, M. & Hiramatsu, K. Beta-hemolysin promotes skin colonization by Staphylococcus aureus. *J. Bacteriol.* **195,** 1194–1203 (2013).

99. Verkaik, N. J. *et al.* Anti-Staphylococcal Humoral Immune Response in Persistent Nasal Carriers and Noncarriers of *Staphylococcus aureus*. *J. Infect. Dis.* **199,** 625–632 (2009).

100. Le, T., Nadimpalli, M., Wu, J., Heaney, C. D. & Stewart, J. R. Challenges in Estimating Characteristics of Staphylococcus aureus Nasal Carriage Among Humans Enrolled in Surveillance Studies. **6,** 1–6 (2018).

101. Verkaik, N. J. *et al.* Heterogeneity of the humoral immune response following Staphylococcus aureus bacteremia. *Eur. J. Clin. Microbiol. Infect. Dis.* **29,** 509–518 (2010).

102. den Reijer, P. M. *et al.* Characterization of the Humoral Immune Response during Staphylococcus aureus Bacteremia and Global Gene Expression by Staphylococcus aureus in Human Blood. *PLoS One* **8,** (2013).

103. Nadimpalli, M. *et al.* Persistence of livestock-associated antibiotic-resistant Staphylococcus aureus among industrial hog operation workers in North Carolina over 14 days. *Occup. Environ. Med.* **72,** 90–99 (2015).

104. Augustine, S. A. J. *et al.* Developing a Salivary Antibody Multiplex Immunoassay to Measure Human Exposure to Environmental Pathogens. *J. Vis. Exp.* 1–7 (2016). doi:10.3791/54415

105. Augustine, S. A. J. *et al.* Immunoprevalence to Six Waterborne Pathogens in Beachgoers at Boquerón Beach, Puerto Rico: Application of a Microsphere-Based Salivary Antibody Multiplex Immunoassay. *Front. Public Heal.* **5,** (2017).

106. P., B. Secretory immunity with special reference to the oral cavity. *J. Oral Microbiol.* **5,** 1–24 (2013).

107. Brandtzaeg, P. Do salivary antibodies reliably reflect both mucosal and systemic immunity? *Ann. N. Y. Acad. Sci.* **1098,** 288–311 (2007).

108. Food, D. Protocol for spa typing. **37,** 11–12 (2009).

109. Pisanic, N. *et al.* Minimally Invasive Saliva Testing to Monitor Norovirus Infection in Community Settings. *J. Infect. Dis.* 1–9 (2018). doi:10.1093/infdis/jiy638

110. Jacobsson, G., Colque-Navarro, P., Gustafsson, E., Andersson, R. & Möllby, R. Antibody responses in patients with invasive Staphylococcus aureus infections. *Eur. J. Clin. Microbiol. Infect. Dis.* **29,** 715–725 (2010).

111. Finkelstein, M. S., Tanner, M. & Freedman, M. L. Salivary and serum IgA levels in a geriatric outpatient population. *J. Clin. Immunol.* **4,** 85–91 (1984).

112. McGhee, J. R. & Fujihashi, K. Inside the Mucosal Immune System. *PLoS Biol.* **10,** 1–5 (2012).

113. Burian, M., Wolz, C. & Goerke, C. Regulatory adaptation of staphylococcus aureus during nasal colonization of humans. *PLoS One* **5,** (2010).

114. Stulik, L. *et al.* α-hemolysin activity of methicillin-susceptible Staphylococcus aureus predicts ventilator-associated pneumonia. *Am. J. Respir. Crit. Care Med.* **190,** 1139–1148 (2014).

115. Jenkins, A. *et al.* Differential Expression and Roles of. *MBio* **6,** 1–10 (2015).

116. D.E., T. *et al.* Staphylococcus aureus alpha-toxin is conserved among diverse hospital respiratory isolates collected from a global surveillance study and is neutralized by monoclonal antibody MEDI4893. *Antimicrob. Agents Chemother.* **60,** 5312–5321 (2016).

117. Rozemeijer, W. *et al.* Evaluation of Approaches to Monitor Staphylococcus aureus Virulence Factor Expression during Human Disease. *PLoS One* **10,** 1–14 (2015).

118. Hau, S. J., Sun, J., Davies, P. R., Frana, T. S. & Nicholson, T. L. Comparative prevalence of immune evasion complex genes associated with β-hemolysin converting bacteriophages in mrsa st5 isolates from swine, swine facilities, humans with swine contact, and humans with no swine contact. *PLoS One* **10,** 1–13 (2015).

119. Stegger, M. *et al.* Rapid differentiation between livestock-associated and livestock-independent staphylococcus aureus CC398 clades. *PLoS One* **8,** 1–5 (2013).

120. Sung, J. M. L., Lloyd, D. H. & Lindsay, J. A. Staphylococcus aureus host specificity: Comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology* **154,** 1949–1959 (2008).

121. Tang, Y. *et al.* Commercial biocides induce transfer of prophage Φ13 from human strains of Staphylococcus aureus to livestock CC398. *Front. Microbiol.* **8,** 1–11 (2017).

122. Pisanic, N. *et al.* Development of an oral fluid immunoassay to assess past and recent hepatitis E virus (HEV) infection. *J. Immunol. Methods* **448,** 1–8 (2017).

123. Wamel, W. Van, Belkum, A. Van & Hamat, R. A. Differences in humoral immune response between patients with or without nasal carriage of Staphylococcus aureus. 451–458 (2017). doi:10.1007/s10096-016-2817-3

124. Croze, M. *et al.* Serum antibodies against Panton – Valentine leukocidin in a normal population and during Staphylococcus aureus infection. *Clin. Microbiol. Infect.* **15,** 144–148 (2009).

CURRICULUM VITAE

Pranay R. Randad, Ph.D.

prandad1@jhmi.edu

3921 Sweetbriar Lane, Frederick MD, 21704

EDUCATION

Johns Hopkins Bloomberg School of Public Health **Present**
Ph.D., Environmental Health and Engineering

Georgetown University **August 2013**
M.S., Biochemistry and Molecular Biology

University of Maryland, College Park **May 2012**
B.S., Physiology and Neurobiology

RESEARCH EXPERIENCE

Johns Hopkins Bloomberg School of Public Health, Environmental Health and Engineering (Baltimore, MD) **2015-Present**
Ph.D. Candidate; Advisor: Christopher D. Heaney, Ph.D.
Transmission dynamics, pathogenicity, and biomarkers of livestock-associated *Staphylococcus aureus* among swine workers and swine raised on concentrated animal feeding operations in the United States.

- Conducted whole genome sequence analysis (WGSA) to investigate transmission dynamics and pathogenicity of multidrug-resistant *S. aureus* commonly contracted by swine workers
- Developed non-invasive immunological biomarkers of *S. aureus* colonization to study early biological effects of exposure to livestock-associated and multidrug-resistant *S. aureus* in humans
- Presented on research findings to scientific audiences and published research findings in peer-reviewed scientific literature

MedStar Health Research Institute, Firefighters' Burn and Surgical Research Laboratory (Washington, DC) **2010-2013**
Graduate research assistant; Advisor: Jeffrey W. Shupp, MD
Pathophysiologic basis of burn wound progression

- Advanced *in-vitro* and *in-vivo* projects aimed at understanding MRSA infection and cutaneous burn-wound healing, the systems biology of infected burn-wound injury at the host-pathogen interface, and the role of microbiome in cutaneous wound healing.

TEACHING EXPERIENCE

Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) **2016-2018**

- Graduate teaching assistant, Introduction to Public Health Emergency Preparedness

National Science Foundation Teaching Fellow (Prince George's County, MD) **2011**

- Physics instructor, Advanced Placement Physics, Eleanor Roosevelt High School

Aston Language Center (Wuhu, China) **2011**

- English teacher for students in China

RELATED PROFESSIONAL EXPERIENCE

QIAGEN, Molecular Diagnostics (Frederick, MD) **2013-2015**
Associate Scientist

- Collaborated with large R&D division to design and develop cutting edge molecular diagnostic tools for clinical application
- Successfully advanced DNA sequencing based diagnostic tools from R&D to Product Development stages of product development pipeline

AWARDS AND HONORS

- The Johns Hopkins NIOSH Education and Research Center, Pilot Project Research Training Award; $10,000 (2015)
- Johns Hopkins University Center for Global Health, Global Health Established Field Placement Award; $5,000 (2016)
- Environmental Health and Engineering Student Pilot Project Award; $3,000 (2017)
- Georgetown University, Academic Excellence Award (2013)
- Georgetown University, Excellence in Biochemistry Internship (2013)
- Werner H. Kirsten Student Intern Cancer Research Training Award (2008)
- Maryland Distinguished Academic Scholar (2008)

PEER REVIEWED PUBLICATIONS

Randad P.R., Dillen C.A., Ortines R.O., Mohr D., Aziz M., Price L.B., Kaya H., Larsen J., Carroll K.C., Smith T.C., Miller L.S., Heaney C.D. Comparison of livestock-associated and community-associated *Staphylococcus aureus* pathogenicity in a mouse model of skin and soft tissue infection. Scientific Reports. *Accepted, 2019.*

Randad P.R., Kaya H., Larsen J., Pisanic N., Smith T.C., Davis M.F., Milstone A.M., Miller L.S., Heaney C.D. Livestock-associated *Staphylococcus aureus* CC9: Population structure and evidence of transmission between pigs raised on industrial hog operations and humans in North Carolina, USA. *In preparation, 2019.*

Randad P.R., Pisanic N., Davis M.F., Milstone A.M., Miller L.S., Heaney C.D. Non-invasive immunological biomarkers of *S. aureus* exposure among swine workers in the United States. *In preparation, 2019.*

Peng Q., Vijaya Satya R., Lewis M., **Randad P.**, Wang Y. Reducing amplification artifacts in high multiplex amplicon sequencing by using molecular barcodes. BMC Genomics. 2015 August; 16:589.

Mino, M.J., Ortiz, R.T., **Randad, P.R.**., Moffatt, L.T., Jordan, M.H., Shupp, J.W. Localization of Superantigen Virulence Factors in Kidney Tissue of Animals with *Staphylococcus aureus*-Infected Burn Wounds**.** *J Burn Care Res*. 2013 Jan-Feb;34(1):142-50

Shupp, J.W., Ortiz, R.T., Moffatt, L.T., Jo, D.Y., **Randad, P.R.**, Mauskar N.A., Mino, M.J., Amundsen, B.A., Jordan, M.H. Treatment with an Oxazolidinone Antibiotic Inhibits TSST-1 Production in MRSA-Infected Burn Wounds. *J Burn Care Res*. 2013 Jan.

Shupp, J.W., Moffatt, L.T., Nguyen, T., Ramella-Roman, J.C., Hammamieh, R., Miller, S.A., Leto, E.J., Jo, D.Y., **Randad, P.R.**, Jett, M., Jeng, J.C., Jordan, M.H. Examination of local and systemic in vivo responses to electrical injury using an electrical burn delivery system. *J Burn Care Res*. 2012 Jan;33(1):118-29.

PRESENTATIONS

Randad P.R., Dillen C.A., Ortines R.O., Mohr D., Aziz M., Price L.B., Kaya H., Larsen J., Carroll K.C., Smith T.C., Miller L.S., Heaney C.D. Comparison of livestock-associated and community-associated *Staphylococcus aureus* pathogenicity in a mouse model of skin and soft tissue infection. Presented at International Symposium on *Staphylococci* and *Staphylococcal* infections, Copenhagen, Denmark, August 2018.

Randad P.R., Kaya H., Larsen J., Pisanic N., Smith T.C., Davis M.F., Milstone A.M., Miller L.S., Heaney C.D. Livestock-associated *Staphylococcus aureus* CC9: Population structure and evidence of transmission between swine and swine-workers and their household contacts in the United States. Oral presentation at Johns Hopkins Environmental Health and Engineering PhD Student Seminar, October 2018.

Carney B.C., **Randad P.R.**, Ortiz R.T., Moffatt L.T., Shupp M.D. Colonizing Bacteria Impacts Local Host Response to Inflammation and Wound Healing for Burn Wounds: A Preliminary Burn Wound Microbiome Analysis. Presented at Annual American Burn Association Conference, Boston MA, March 2014.

Randad P.R., Ortiz R.T., Jo D.Y., Moffatt L.T., Jordan M.H., Shupp M.D. Transcriptomic Perturbations in the Local Innate Immune Response Caused by *Staphylococcus Aureus* Infected Burn Wounds. Presented at MedStar Health Research Annual Symposium, Columbia MD, March, 2013.

Randad P.R., Ortiz R.T., Jo D.Y., Moffatt L.T., Jordan M.H., Shupp M.D. Local Toll-like Receptor Transcript Regulation in Response to Staphylococcus Aureus Infected Burn Wounds. Presented at Georgetown University Medical Center, Washington D.C., July 2013.

REFERENCES

Dr. Christopher Heaney

Director, Johns Hopkins Environmental Health Microbiology and Immunology Laboratory (EHMIL)

Assistant Professor

615 N. Wolfe Street

Room W7033B

Baltimore, Maryland 21205

cheaney1@jhu.edu

443-287-4989

Relationship: PhD Advisor

Dr. Jeffrey W. Shupp, MD

Attending Surgeon and Director of Burn Research at MedStar Washington Hospital Center

110 Irving St NW #3b

Washington, DC 20010

(202) 877-7347

Jeffrey.w.shupp@medstar.net

Relationship: PI/Mentor for 3 years

Dr. Cynthia Marie Rosenthal

Director of M.S. Biochemistry and Molecular Biology

Professor (202) 687-1088

simbulac@georgetown.edu

Relationship: Graduate Director and Professor at Georgetown University